UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE MEDICINA
DEPARTAMENTO DE BIOQUÍMICA

INTERRELACIÓN ENTRE PROTEÍNAS ASOCIADAS A UNIONES INTERCELULARES ESTRECHAS, POLARIDAD DE LOS HEPATOCITOS Y EL VIRUS DE LA HEPATITIS C

IGNACIO BENEDICTO ESPAÑOL

Madrid, 2011

Departamento de Bioquímica
Facultad de Medicina
Universidad Autónoma de Madrid

INTERRELACIÓN ENTRE PROTEÍNAS ASOCIADAS A UNIONES INTERCELULARES ESTRECHAS, POLARIDAD DE LOS HEPATOCITOS Y EL VIRUS DE LA HEPATITIS C

Ignacio Benedicto Español
Licenciado en Bioquímica

Directores de Tesis
Manuel López Cabrera
Pedro Lorenzo Majano Rodríguez

Unidad de Biología Molecular, Hospital Universitario de la Princesa,
Instituto de Investigación Sanitaria Princesa (IP), Madrid.
CIBERehd, Instituto de Salud Carlos III, Madrid.

AGRADECIMIENTOS

En primer lugar me gustaría agradecer a Manuel la oportunidad que me brindó de iniciarme en el mundo de la Ciencia, así como toda la confianza que ha depositado en mí desde el principio. Siempre ha hecho por mí todo lo que ha estado en su mano y ha aportado energía, optimismo y soluciones cuando los problemas han intentado dejarse ver.

Gracias a Manolo, mi profesor de Física, Química y Matemáticas en EGB, porque despertó mi curiosidad por las cosas y con él empecé a intentar comprender lo que pasa a mi alrededor. Esta tesis se empezó a gestar en sus clases, y a él está dedicada.

Estaré en deuda eternamente con Marta, quien me enseñó casi todo lo que he aprendido en el laboratorio y fue un gran ejemplo de cómo se debe trabajar: con talento, ganas, mimo, orden, paciencia y constancia. También con Enrique, que con su pragmatismo y buenas ideas me ayudó a aprender a ser más eficaz. Y en realidad con todas las personas con las que he compartido laboratorio, de las que siempre he recibido su ayuda, sus sabios consejos y su buen humor.

Estos últimos años he tenido la enorme suerte de trabajar con Paqui y Pedro. Sus ideas y habilidades, su gran esfuerzo y trabajo, y su permanente compañerismo y buen rollo son los ingredientes básicos sin los cuales esta tesis no hubiera sido posible.

Quiero agradecer también a Ricardo su apoyo incondicional, su entusiasmo y su espíritu alegre y colaborador. Si está él, las cosas son más fáciles y todo es más agradable.

Me gustaría dar las gracias a Martin A. Schwartz por permitirme trabajar durante un tiempo con su grupo en la Universidad de Virgina, donde aprendí un montón de cosas y pude experimentar cómo funciona un laboratorio americano. También a François-Loïc Cosset y a toda la gente de la Universidad de Lyon, que me hicieron sentir allí como en casa y que, junto con Rafa Aldabe del CIMA de Pamplona, han aportado la tecnología y experiencia imprescindibles para llevar a cabo todo nuestro trabajo relacionado con HCV.

Mis amigos, que siempre han estado ahí, también son responsables de esta tesis. En especial Los Chicos, porque ir con ellos a tocar es lo más divertido del mundo y hace que te olvides de cualquier preocupación.

Quiero expresar mi más profundo agradecimiento a mi familia y en especial a mis padres, por ser como son, por confiar en mí y apoyarme en todo lo que hago. Sois los mejores.

Y a Marta, porque la vida a su lado es mil millones de veces mejor.

RESUMEN

Las uniones intercelulares estrechas o “tight junctions” (TJs) son complejos macromoleculares que posibilitan el mantenimiento estructural y funcional de la polaridad de las células epiteliales. Además, constituyen la primera barrera defensiva que impide la entrada de la mayoría de agentes infecciosos al interior del organismo. Sin embargo, algunos patógenos son capaces de modificar estas estructuras con el objetivo de penetrar en el huésped, contribuyendo así al desarrollo de determinados aspectos de la patología asociada a la infección. Así, este trabajo muestra que el virus de la hepatitis C (HCV) induce la deslocalización de las proteínas asociadas a TJs ocludina, claudina-1 y ZO-1, así como la alteración de sus funciones características, lo que podría explicar alguna de las disfunciones hepáticas provocadas por el virus. Además, se pudo observar que HCV induce la acumulación intracelular de ocludina, donde interacciona con la proteína E2 de la envuelta viral. Esta observación dio pie a estudiar el posible papel de ocludina como co-receptor de HCV, lo que fue confirmado en varios sistemas experimentales. Finalmente, dada la estrecha relación observada entre HCV y las proteínas asociadas a TJs, se desarrolló un sistema de cultivo tridimensional (3D) que permite el estudio del ciclo completo de HCV en un contexto donde las células presentan la polaridad típica de los hepatocitos, lo que puede afectar de forma notable diferentes fases del ciclo viral. De hecho, se observó que las partículas infectivas generadas en 3D, en comparación con el virus obtenido en condiciones de cultivo tradicionales, eran más parecidas a las existentes en pacientes en términos de densidad e infectividad específica. En resumen, este trabajo pone de manifiesto la interrelación existente entre HCV y las TJs en lo que se refiere tanto a alteraciones celulares promovidas por el virus como a la participación de proteínas de la célula diana en el ciclo viral, y propone un nuevo modelo para el estudio de la interacción virus-huésped en un contexto más fisiológico que el proporcionado por los sistemas basados en líneas celulares empleados hasta la fecha.

SUMMARY

Tight junctions (TJs) are macromolecular complexes that enable the maintenance of structural and functional polarity in epithelial cells. In addition, they constitute the first defensive barrier that prevents the entry of most infectious agents into the body. However, some pathogens are able to modify these structures in order to enter the host cell, contributing to some aspects of the infection-associated pathology. Herein we have shown that hepatitis C virus (HCV) promotes the mislocalization of the TJ-associated proteins occludin, claudin-1 and ZO-1 as well as the alteration of their functions, being a possible cause of some of the hepatic disfunctions occasionated by the virus. Moreover, it could be observed that HCV induced the intracellular accumulation of occludin, where it interacted with the viral envelope protein E2. This observation led to study the possible role of occludin as a HCV co-receptor, hypothesis that was confirmed in several experimental systems. Finally, given the tight relation observed between HCV and TJ-associated proteins, we developed a three dimensional (3D) culture system that allowed the study of the complete HCV cycle in a context of hepatocyte-like polarization, fact that could possibly affect different steps of the viral cycle. Indeed, it was observed that, compared to virions obtained from traditional cultures, 3D-generated viral particles shifted towards lower densities that exhibited a higher associated specific infectivity, resembling HCV virions present in natural infection. In summary, this study reveals the interplay between HCV and TJs in terms of both virus-induced cellular alterations and the involvement of host proteins in the viral cycle, and proposes a new model for the study of virus-host interactions that provides a more physiological context than currently employed cell line-based systems.

ÍNDICE

ABREVIATURAS

HCV, virus de la hepatitis C
IRES, secuencia interna de entrada al ribosoma
ER, retículo endoplásmico
TJ, unión intercelular estrecha o “tight junction”
VLDLs, lipoproteínas de muy baja densidad
MTP, proteína de transferencia microsomal
LDLs, lipoproteínas de baja densidad
LDL-R, receptor de lipoproteínas de baja densidad
SR-BI, receptor scavenger clase B tipo I
LEL, dominio extracelular de mayor tamaño
siRNA, RNA pequeño de interferencia
shRNA, RNA pequeño de horquilla
uPA-SCID, activador del plasminógeno tipo urokinasa-inmunodeficiencia combinada severa
TEMs, microdominios enriquecidos en tetraspaninas
HDLs, lipoproteínas de alta densidad
CHO, células de ovario de hámster chino
PHHs, hepatocitos primarios humanos
HCVser, HCV procedente del suero de pacientes infectados
HCV-LPs, partículas similares a HCV o “HCV-like”
HCVpp, pseudopartículas de HCV
GFP, proteína verde fluorescente
HCVcc, HCV generado en cultivos celulares
CAR, receptor de Coxsackievirus y adenovirus
ESAM, molécula de adhesión específica de células endoteliales
JAMs, moléculas de adhesión intercelular
ZO-1, ZO-2 y ZO-3, zonula occludens 1, 2 y 3
SVDV, virus de la enfermedad vesicular porcina
CVB, virus Coxsackie tipo B3
DAF, proteína “decay-accelerating factor”
VEGF, factor de crecimiento del endotelio vascular
HIV, virus de la inmunodeficiencia humana
3D, tridimensional

INTRODUCCIÓN

INTRODUCCIÓN

1. EL VIRUS DE LA HEPATITIS C

1.1 Patología, terapéutica y estructura viral.

El virus de la hepatitis C (HCV) fue identificado en 1989 como el agente causante de la hepatitis no-A no-B (215), y su principal vía de transmisión es el contacto sanguíneo (176). En la actualidad, HCV infecta a tres millones de nuevos individuos anualmente, siendo la población total infectada alrededor de 150 millones de personas (aproximadamente el 3% de la población mundial) (78). Más del 80% de las infecciones agudas por HCV evolucionan hacia un estado crónico que eventualmente provoca el desarrollo de fibrosis hepática, cirrosis y carcinoma hepatocelular (176). El daño hepático causado por la infección crónica por HCV no es debido a la destrucción directa del hepatocito por el virus, sino que es el resultado de procesos inflamatorios persistentes a consecuencia de una respuesta inmune prolongada y no completamente efectiva (89). La infección por HCV es la causa más frecuente de transplante hepático en Europa y Estados Unidos, lo que la convierte en un importante problema de salud pública (176).

Actualmente no existe ninguna vacuna profiláctica o terapéutica eficaz contra HCV, y el tratamiento estándar consistente en la administración combinada de interferón alfa pegilado y ribavirina sólo es efectivo en la mitad de los pacientes infectados con HCV de genotipo 1 (el más común en Europa, Japón y América del Norte) y presenta severos efectos secundarios (78). Recientemente, se ha descrito que la eliminación del virus, tanto espontánea como en respuesta a este tratamiento, está asociada a una serie de polimorfismos en el promotor del gen *IL28B* (143, 201, 202), lo que puede ser importante a la hora de decidir la conveniencia de la terapia. En búsqueda de una alternativa al tratamiento estándar, se están llevando a cabo ensayos clínicos en fase III con compuestos antivirales específicos para HCV (78). Asimismo, la identificación del microRNA hepático miR-122 como una posible diana terapéutica eficaz contra HCV (119) ha propiciado el inicio de nuevos ensayos clínicos en esa dirección (94).

HCV es un virus con envuelta, perteneciente a la familia *Flaviviridae*, que puede infectar a humanos, chimpancés y tupayas (7, 174, 204). El genoma de HCV, una cadena de RNA de polaridad positiva de 9,6 kilobases, codifica para una poliproteína

de ~3000 aminoácidos que es procesada por proteasas virales y celulares para dar lugar a diez proteínas maduras, que incluyen tres proteínas estructurales [la proteína de la cápsida (core) y dos glicoproteínas de envuelta (E1 y E2)], la proteína p7 y las proteínas no estructurales (NS2, NS3, NS4A, NS4B, NS5A y NS5B) (170) (Fig. 1).

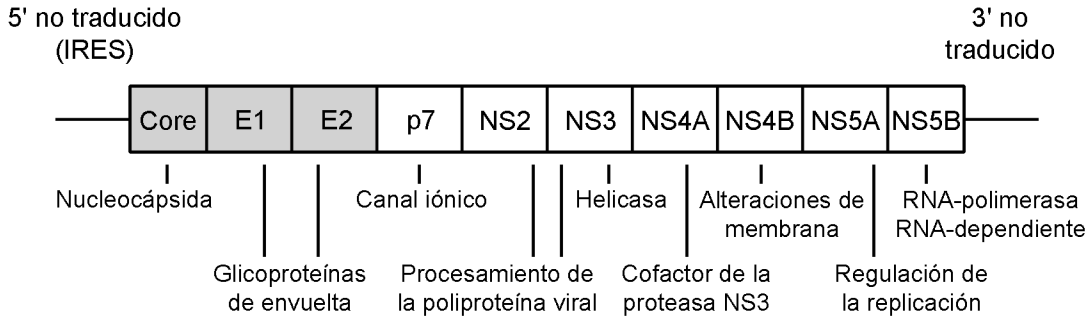


Figura 1. Esquema del genoma de HCV. El RNA de HCV codifica una única poliproteína que, tras su procesamiento mediante proteasas celulares y virales, da lugar a las proteínas estructurales (gris) y no estructurales. Se indica la presencia de los extremos 5' y 3' no traducidos, la secuencia interna de entrada al ribosoma (IRES) y las principales funciones de cada proteína en el ciclo viral (56).

Además, se han descrito formas de core resultantes de un marco de lectura alternativo denominadas ARFPs, aunque sus funciones son desconocidas (31). Existen seis genotipos principales de HCV con aproximadamente un 70% de homología, y en 2008 se descubrió un séptimo genotipo (170). Además, debido a la alta tasa de replicación del virus y al elevado número de errores cometidos por la polimerasa viral, el individuo infectado presenta variantes de HCV conocidas como cuasiespecies (225). Esta capacidad mutagénica permite al virus adaptarse a diferentes condiciones ambientales y escapar a la respuesta inmune del huésped, lo que favorece la persistencia viral (225).

Hasta la fecha, no se ha podido resolver la estructura tridimensional de las proteínas estructurales de HCV, ni tampoco se han obtenido imágenes de alta resolución de la partícula viral. Sin embargo, mediante microscopía electrónica de transmisión, se ha observado que el virus presenta un diámetro aproximado de 60 nm (105, 124, 177, 224) y está constituido por una nucleocápsida, que contiene el genoma viral compactado por la proteína core, rodeada por una bicapa lipídica donde se encuentran ancladas las proteínas de la envuelta E1 y E2 (16, 169). E1 y E2 son proteínas transmembrana tipo I con un ectodominio N-terminal y un corto dominio

transmembrana C-terminal, y son fundamentales para la entrada de HCV en el interior del hepatocito (16, 170). Durante la síntesis de E1 y E2 en la célula infectada, sus ectodominios están situados en el lumen del retículo endoplásmico (ER) y sus dominios transmembrana se encuentran anclados en la membrana de dicho compartimento, mediando la formación de heterodímeros no covalentes entre ambas proteínas (95). Sin embargo, en la superficie del virus, E1 y E2 interaccionan entre sí formando complejos covalentes estabilizados por puentes disulfuro (212). Sus ectodominios se encuentran altamente glicosilados, lo que es importante para su correcto plegamiento y para la entrada del virus en la célula huésped (81), así como para evadirse del sistema inmune enmascarando epítopos inmunogénicos (96).

En el suero de los pacientes, HCV se encuentra como una población heterogénea de partículas virales de densidades comprendidas entre 1,03 y 1,34 g/ml (10). En este rango, se ha detectado RNA viral asociado a diferentes tipos de partículas incluyendo nucleocápsidas sin envuelta (136), exosomas (vesículas que contienen RNA viral, proteínas de la envuelta y CD81) (140) y lipoviropartículas (9, 162). Éstas últimas representan alrededor del 40% del RNA viral en plasma (82), y están compuestas por la nucleocápside viral, las proteínas de la envuelta y lipoproteínas ricas en triglicéridos que contienen las apolipoproteínas ApoB y ApoE (9, 53, 162) responsables de la baja densidad de este tipo de partículas. De hecho, se ha observado que la densidad de las partículas de HCV *in vivo* es muy dinámica y depende de la concentración en plasma de lipoproteínas ricas en triglicéridos (53, 63). A pesar de que se ha sugerido que HCV y las lipoproteínas se pueden asociar extracelularmente, habiéndose generado ambas partículas de forma independiente (63), múltiples estudios han demostrado que la morfogénesis viral está íntimamente relacionada con la biosíntesis de lipoproteínas de muy baja densidad (VLDLs) (27, 47, 76, 100, 101, 103), estando implicadas en ambos procesos la proteína de transferencia microsomal (MTP), ApoE y ApoB, aunque el papel de ésta última en el ensamblaje de HCV resulta controvertido (47, 103). Además, se ha descrito que los precursores intracelulares de HCV poseen una mayor densidad que los viriones que se encuentran en el medio extracelular (77). Estos datos sugieren que durante la generación de las partículas virales, éstas se asocian con las lipoproteínas a lo largo de la ruta de maduración y secreción de VLDLs.

Se ha observado una relación inversamente proporcional entre densidad e infectividad de la partícula viral *in vitro* e *in vivo* (2, 24, 30, 128, 175). Además, es posible inhibir la infección por HCV *in vitro* mediante el uso de anticuerpos dirigidos frente a ApoB (2, 9, 11) y ApoE (2, 9, 47, 165), así como bloqueando o reduciendo la expresión del

receptor de lipoproteínas de baja densidad (LDL-R) (2, 9, 30, 155). Asimismo, se ha observado que las VLDLs y LDLs bloquean la infección por HCV, posiblemente compitiendo por el sitio de unión al LDL-R e impidiendo la unión eficaz de la partícula viral a la célula (9, 155). Por último, se ha descrito que la delipidación de las lipoviropartículas mediante la lipoproteína lipasa inhibe la infección por HCV *in vitro* (196). En conjunto, estas observaciones indican que las lipoproteínas presentes en la partícula viral participan de forma importante en el proceso de entrada de HCV en la célula huésped. Además, podrían estar implicadas en la protección del virus frente al sistema inmune mediante el enmascaramiento de los epítopos de las proteínas de envuelta reconocidos por los anticuerpos neutralizantes.

Existen múltiples evidencias de que HCV es un virus con tropismo hepático. Sin embargo, la alta frecuencia de manifestaciones extrahepáticas vinculadas a la infección crónica ha hecho cuestionarse si HCV es capaz de infectar y replicarse *in vivo* en células distintas a los hepatocitos (126). Por ejemplo, la infección crónica por HCV se asocia frecuentemente a desórdenes linfoproliferativos (230) y anomalías del sistema nervioso central (98). Algunos estudios sugieren que el virus puede infectar células linfoides y tejidos neuronales (64, 67, 219, 230), aunque otros grupos han puesto en duda que HCV tenga la capacidad de replicarse activamente en estas células (35, 118, 139, 198).

Por otra parte, se ha observado que la población de cuasiespecies virales presentes en el hígado es significativamente diferente de la que se encuentra asociada a las lipoviropartículas del suero, lo que sugiere que éstas últimas pueden tener una procedencia extrahepática (51). Además del hígado, el intestino también tiene la capacidad de sintetizar lipoproteínas (80), por lo que se ha especulado con la posibilidad de que pueda contribuir a la generación de lipoviropartículas. De hecho, se ha detectado la presencia de proteínas virales en los enterocitos de pacientes infectados (51). Además, se ha conseguido aislar lipoviropartículas que contienen la apolipoproteína ApoB-48, producida específicamente en el intestino (53), aunque por otro lado se ha propuesto que esta asociación puede ocurrir después de que la partícula viral y la apolipoproteína se hayan generado de forma independiente (63). Por todo ello, el posible tropismo extrahepático de HCV sigue siendo una cuestión controvertida que precisa ser dilucidada para identificar posibles reservorios virales que constituyan dianas potenciales para la intervención terapéutica.

1.2 El ciclo viral de HCV

La infección comienza con la adhesión de la partícula viral a la célula huésped mediante interacción con moléculas de la superficie celular (225) (Fig. 2). Posteriormente, el virus penetra en la célula a través de endocitosis dependiente de clatrina (29, 150) y se aloja temporalmente en endosomas tempranos (150). Seguidamente, la acidificación del endosoma provoca la fusión de la envuelta del virus

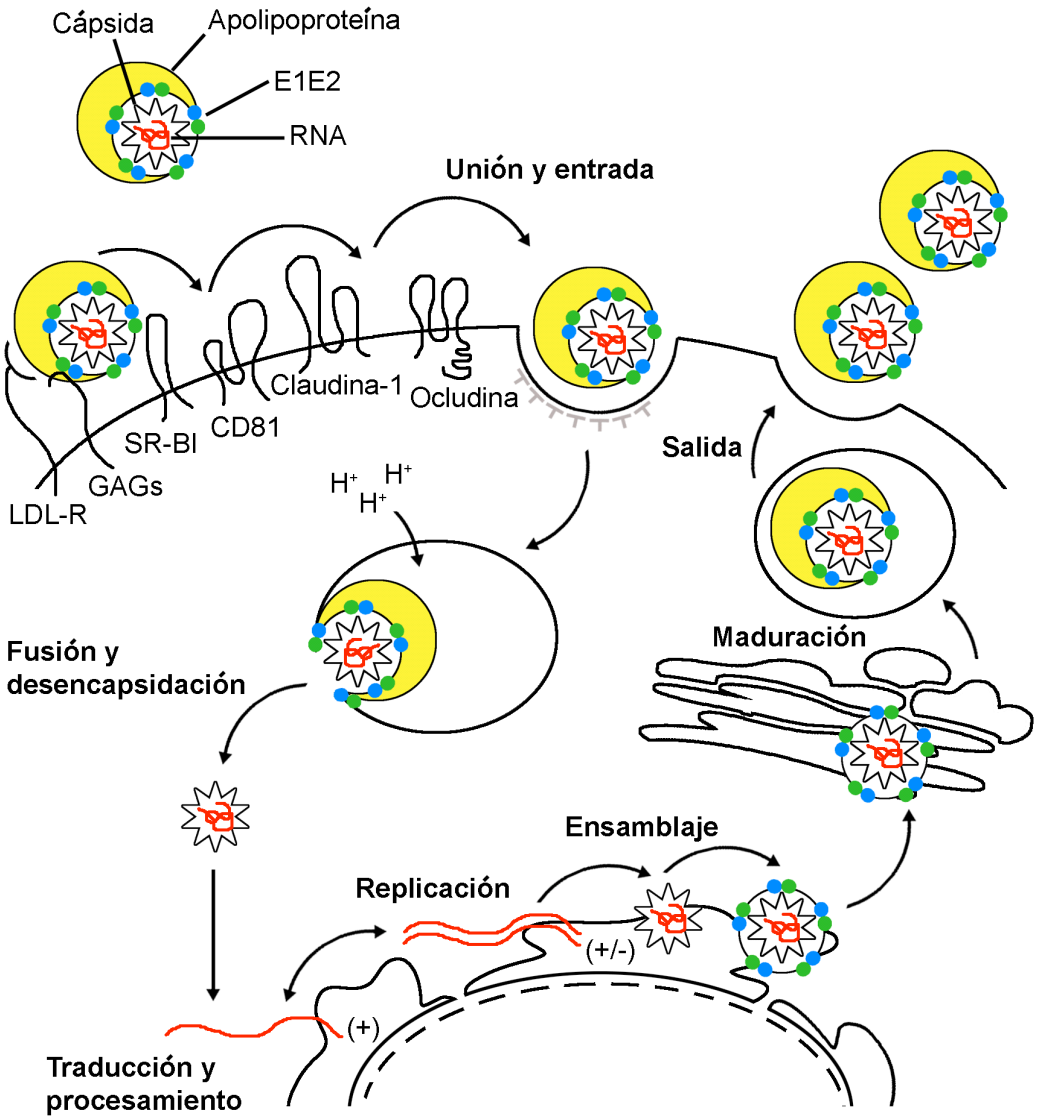


Figura 2. Ciclo viral de HCV. La partícula viral interacciona con los factores de anclaje y co-receptores celulares y se internaliza vía endocitosis mediada por clatrina. La acidificación del endosoma temprano provoca su fusión con la envuelta viral. Tras la desencapsidación, el RNA genómico de polaridad positiva (+) se traduce dando lugar a una única poliproteína, que es procesada por proteasas celulares y virales. Tras la replicación se produce el ensamblaje y maduración de la progenie viral, que finalmente sale de la célula.

con la membrana endosomal (22, 99) liberando así en el citosol la nucleocápsida con el genoma de HCV. Tras su desencapsidación, el RNA viral se traduce y procesa para dar lugar a las proteínas estructurales y no estructurales. Éstas últimas posibilitan el proceso de la replicación viral, que ocurre en membranas derivadas de ER (12, 58, 85). El ensamblaje de nuevos viriones comienza en la cara citosólica del ER en contacto con gotas lipídicas, después de lo cual las partículas nacientes ingresan en el lumen del ER (104). Finalmente, la progenie viral sale de la célula después de su maduración, a través de un proceso que se cree está íntimamente ligada a la vía secretora y a la producción de VLDLs (104). Así, estos nuevos virus secretados están listos para infectar otras células del entorno. Alternativamente, la transmisión del HCV puede ocurrir mediante la transferencia directa del virus desde la célula infectada a la célula contigua mediante un mecanismo no totalmente caracterizado que podría tener lugar sin la liberación de la partícula viral al medio extracelular (33, 203, 220), lo que posibilitaría al virus escapar de los anticuerpos neutralizantes del sistema inmune.

1.3 Entrada de HCV en el hepatocito

1.3.1 Unión a la superficie celular

In vivo, se cree que HCV accede a los hepatocitos penetrando en el hígado mediante la sangre sinusoidal. En línea con esta hipótesis, se ha descrito que L-SIGN, una proteína expresada en las células endoteliales del sinusoide hepático, se une a la proteína viral de envuelta E2 (75, 133) y facilita la transinfección de los hepatocitos *in vitro* (43, 132). El mismo fenómeno se ha observado con la proteína DC-SIGN expresada en células de Kupffer, macrófagos residentes en el sinusoide hepático (43, 132). Por ello, se piensa que HCV puede utilizar las células del sinusoide hepático como anclaje inicial en el hígado, para posteriormente infectar los hepatocitos subyacentes.

El primer paso en la entrada de HCV en el hepatocito es la unión del virus a la célula diana. Se ha descrito que esta interacción ocurre mediante el glicosaminoglicano sulfato de heparano (17, 18, 114), un polisacárido lineal presente en los proteoglicanos de la superficie celular que también posibilita los primeros contactos de otros virus con sus células huésped (17). Por otra parte, LDL-R también participa en la interacción inicial de HCV con el hepatocito, probablemente mediante la unión a lipoproteínas asociadas a la partícula infectiva (2, 155).

1.3.2 Interacción con los co-receptores e internalización

Una vez que el virus entra en contacto con el hepatocito, una serie de proteínas presentes en la membrana celular intervienen en la internalización de la partícula viral: la tetraspanina CD81, el receptor scavenger clase B tipo I (SR-BI) y las proteínas asociadas a TJs claudina-1 y ocludina (ver apartado 2.3 y Anexo 2).

CD81, perteneciente a la familia de las tetraspaninas, presenta cuatro dominios transmembrana y extremos N y C-terminales intracelulares (48). Sus funciones fisiológicas se conocen sólo parcialmente: forma parte del complejo receptor del linfocito B y participa en la fusión del óvulo con el espermatozoide, así como en la regulación de la composición celular del cerebro (48). Además, es necesario para la entrada de los esporozoitos de *Plasmodium falciparum*, agente causante de la malaria, en los hepatocitos del huésped (197). CD81 fue la primera molécula identificada como posible receptor de HCV al describirse la interacción de su dominio extracelular de mayor tamaño (LEL) con la proteína E2 soluble (172). Numerosos estudios posteriores han confirmado la interacción HCV-CD81, proporcionando además información detallada acerca de los aminoácidos de la glicoproteína viral E2 y del dominio LEL de CD81 implicados en esta interacción (28, 52, 65, 167, 168). Mediante el uso de diferentes sistemas experimentales *in vitro*, se ha observado que anticuerpos y siRNAs frente a CD81, así como una forma soluble del dominio LEL, inhiben la entrada de HCV en las células diana (20, 43, 99, 106, 123, 156, 216, 227, 229). Asimismo, se ha demostrado la eficacia de un anticuerpo anti-CD81 como protección frente a la infección por HCV *in vivo* en el modelo de ratón uPA-SCID con hígado humanizado (152). Por otra parte, se han caracterizado anticuerpos anti-E2 que bloquean la infección interfiriendo en la unión E2-CD81 (40, 166). Además, se ha determinado que existe un nivel mínimo de CD81 en membrana por debajo del cual la entrada viral no es eficiente (6, 113), poniendo de manifiesto la importancia de CD81 en el proceso infectivo.

A pesar de que el mecanismo por el cual CD81 posibilita la entrada viral no se conoce con exactitud, algunos datos parecen sugerir que no es necesaria su asociación con microdominios enriquecidos en tetraspaninas (TEMs) para que funcione como co-receptor del virus (183). Además, varios estudios sugieren que CD81 actúa en una fase de la infección posterior a la unión inicial del virus a la célula (43, 59, 114). Por otra parte, se ha observado que aminoácidos localizados en dominios transmembrana e intracelulares están implicados en la entrada del virus sin participar en la interacción

E2-CD81 (28), sugiriendo que la interacción de CD81 con otras proteínas transmembrana y/o intracelulares puede ser importante en su papel como co-receptor de HCV. Se ha descrito que la unión de E2 soluble con CD81 induce la relocalización del complejo hacia zonas de contacto célula-célula, y que este desplazamiento requiere la remodelación del citoesqueleto de actina (32). Además, se ha observado que durante la internalización de la partícula viral, ésta se asocia con CD81 (41). Estos datos sugieren que el papel de CD81 en la entrada de HCV puede ser el de, una vez unido el virión a la célula, transportarlo hacia zonas de la membrana celular donde pueda ser endocitado junto con el co-receptor. Por último, se ha descrito que la proteína EWI-2wint se asocia con CD81 y bloquea la entrada del virus en la célula inhibiendo la interacción E2-CD81 (184). EWI-2wint no se expresa en los hepatocitos, lo que sugiere que, además de la presencia de receptores específicos, la ausencia de determinadas proteínas puede contribuir al tropismo hepático de HCV.

SR-BI posee dos dominios transmembrana y extremos N y C-terminal intracelulares (182). Fue identificado inicialmente como el principal receptor de lipoproteínas de alta densidad (HDLs) en el hígado (1). Mediante un proceso denominado transporte selectivo de lípidos, los ésteres de colesterol presentes en la partícula de HDL unida a SR-BI se incluyen en la membrana celular (42). La observación de que SR-BI es capaz de unirse a la proteína E2 soluble (190) fue el primer paso en su caracterización como co-receptor de HCV (22, 39, 88, 106). Posteriormente se determinó que anticuerpos frente a SR-BI inhibidores de su interacción con E2 bloquean la infección por HCV (39). Además, mutantes de SR-BI con baja afinidad por E2, pero que conservan su capacidad de unirse a HDLs y mediar el flujo de colesterol, no son efectivos como co-receptores de HCV (38). Estos datos sugieren que la interacción de SR-BI con E2 es importante para su función como co-receptor viral. Sin embargo, también se ha sugerido que la interacción de SR-BI con HCV puede estar mediada por ApoB presente en las lipoproteínas asociadas a la partícula viral (135). Se ha observado que la expresión de SR-BI en células de ovario de hámster chino (CHO) es suficiente para que HCVcc se una a la superficie celular (59); por otro lado, estudios cinéticos de inhibición de la infección con anticuerpos bloqueantes parecen indicar que SR-BI actúa durante la unión inicial del virus a la célula (38) así como en una fase posterior coordinada con CD81 (226). Estos datos sugieren que SR-BI puede tener una doble función como co-receptor de HCV, estando implicado en varias fases de la infección.
SR-BI actúa como receptor de múltiples ligandos y posee al menos dos sitios de unión a ligando (182). Dos de ellos, las LDLs oxidadas y el amiloide A del suero, inhiben la entrada de HCV a través de un mecanismo desconocido que parece no implicar la

competición directa por el sitio de unión (37, 120, 214). Sin embargo, la infectividad de HCV se ve incrementada por la presencia de HDLs, el ligando más importante de SR-BI, durante la infección (21, 213). Además, este incremento no se produce al añadir BLT-4, un compuesto que inhibe el transporte selectivo de lípidos sin afectar a la unión de HDLs a SR-BI (21, 213, 214). Por ello, se cree que SR-BI puede facilitar la entrada de HCV de forma indirecta mediante el enriquecimiento de la membrana celular en colesterol; de hecho, se ha observado que la depleción del colesterol celular induce una disminución de los niveles de CD81 en membrana, inhibiendo parcialmente la infección por HCV (106). Finalmente, también se ha sugerido que determinados residuos de la cola citoplasmática C-terminal de SR-BI pueden contribuir a su función como co-receptor de HCV mediante la modulación de su localización en membrana, su tráfico intracelular y su asociación con la proteína scaffold PDZK1 (55, 60).

No se conoce con precisión el papel de cada co-receptor en la entrada viral, ni tampoco si actúan secuencialmente o formando una plataforma macromolecular donde los co-receptores interactúan de manera conjunta con el virus. También se desconoce si alguna de estas moléculas se internaliza junto con la partícula viral. Lo que sí se ha demostrado es que la entrada del virus en la célula ocurre mediante endocitosis dependiente de clatrina (29, 41, 150). También se ha sugerido que este proceso requiere la participación de la red de microtúbulos (185) y los filamentos de actina (41), aunque este último dato es controvertido (185). Además, mediante ensayos de co-localización con marcadores específicos y el uso de dominantes negativos y siRNAs frente a Rab5 y Rab7, se ha determinado que la entrada productiva de HCV requiere su paso por endosomas tempranos pero no tardíos (41, 150) como paso previo a la fusión con la membrana endosomal.

1.3.3 Fusión

Se ha observado que una vez la partícula viral se encuentra en endosomas tempranos, la acidificación de éstos es necesaria para que se produzca su fusión con la envuelta viral (22, 29, 99, 121, 208). Este proceso está favorecido por la presencia de dominios ricos en colesterol y esfingomielina en ambas membranas (4, 91, 106, 121) y parece estar modulado por la densidad de la partícula viral (91). Además, se ha descrito que la fusión depende de las proteínas de envuelta E1 y E2 (91, 121, 122), las cuales presentan ciertas similitudes con las proteínas de fusión de clase II (169). Este tipo de proteínas de fusión se caracterizan por sufrir cambios conformacionales, en respuesta a factores celulares (bajo pH y/o interacción con receptores), que hacen que

se exponga el péptido de fusión permitiendo así su inserción en la membrana celular y posibilitando la fusión (107).

Los virus con envuelta que se internalizan mediante endocitosis son, en general, muy sensibles al pH ácido. El tratamiento a bajo pH induce un cambio conformacional en las proteínas de envuelta que hace que el péptido de fusión se exponga de forma prematura, produciéndose la inactivación del virus (107). Se ha observado que esto no ocurre con HCV, lo que sugiere que sus proteínas de la envuelta necesitan un paso adicional que les confiera sensibilidad a la bajada del pH (150, 208). Además, se ha descrito que la internalización y la fusión de la partícula de HCV ocurren con una diferencia de veinte minutos, en contraste con otros virus donde ambos procesos son prácticamente simultáneos (150). Por todo ello, para que se produzca la fusión virus-endosoma, es posible que sea necesario que las proteínas de la envuelta viral sufran ciertas modificaciones o interaccionen con proteínas celulares que las hagan susceptibles al pH ácido en algún momento entre la unión del virus a la superficie celular y su localización en endosomas (208). De hecho, ensayos de fusión célula-célula sugieren que CD81, claudina-1 y ocludina pueden tener un papel en la fusión de la envuelta viral con la membrana endosomal (26, 59, 109).

1.4 Modelos para el estudio de HCV *in vitro*

Los primeros estudios *in vitro* de la infección por HCV se realizaron mediante la inoculación de hepatocitos primarios humanos (PHHs) con HCV procedente del suero de pacientes infectados (HCVser) (68, 186). Sin embargo, la baja tasa de replicación viral y la ausencia de producción detectable de nuevos virus infectivos dificultan el uso de HCVser como herramienta para estudiar el ciclo viral completo (36). Además, la heterogeneidad existente entre los aislados virales de diferentes pacientes y su incapacidad para infectar productivamente líneas celulares hacen que HCVser no sea el sistema más apropiado para su uso rutinario en investigación y su empleo en análisis “high-throughput” (82). Por ello, con el paso del tiempo se han ido desarrollado sistemas *in vitro* cada vez más sofisticados que permiten el estudio de cada etapa del ciclo viral.

La búsqueda de receptores celulares de HCV comenzó mediante el uso de una forma recombinante y soluble de la glicoproteína E2 de la envuelta viral (172, 190). Sin embargo, las características conformacionales y funcionales de esta versión de E2 pueden diferir en gran medida de las de E2 en su estado natural, anclada en la

membrana del virus formando heterodímeros con E1 (95, 212). Por ello, es probable que E2 soluble no posea todas las propiedades de la proteína nativa, lo que hace que su uso sea limitado y que los resultados obtenidos requieran ser interpretados cuidadosamente. Un paso adelante en el estudio de la entrada viral fue la generación de partículas "HCV-like" (HCV-LPs) producidas en sistemas de expresión de baculovirus en células de insecto (23). Estas partículas sí presentan heterodímeros de E1 y E2 en su envuelta, aunque su estado de glicosilación no refleja fielmente la situación en el contexto de células humanas (36). Además, las HCV-LPs no son secretadas de las células productoras sino que es necesario extraerlas de los compartimentos intracelulares donde quedan retenidas, dificultando así su preparación (36). Estos problemas fueron solucionados en parte con la aparición del sistema de pseudopartículas de HCV (HCVpp) (20, 99), mediante el cual se generan partículas virales que incorporan las glicoproteínas de la envuelta de HCV y son secretadas de

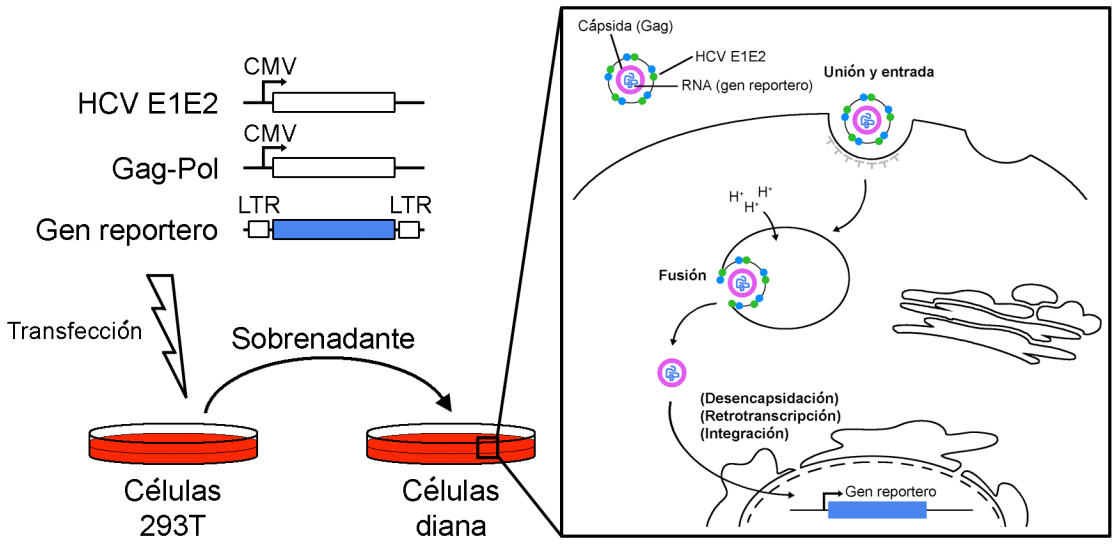


Figura 3. Sistema de HCVpp. Las partículas virales pseudotipadas con las proteínas de envuelta E1 y E2 son válidas para estudiar los primeros pasos de la infección por HCV (unión a la célula diana, endocitosis y fusión). Una vez que se produce la fusión, el resto del ciclo viral (desencapsidación, retrotranscripción e integración en el genoma de la célula huésped) corresponde al del retrovirus empleado. Tras la integración tiene lugar la expresión del gen reportero, que es proporcional a la infectividad de las partículas virales. Las células infectadas no generan nuevos pseudovirus ya que carecen de los genes que codifican la polimerasa y las proteínas estructurales retrovirales. CMV, promotor de citomegalovirus. LTR, repetición terminal larga.

forma natural al medio extracelular (Fig. 3). La producción de HCVpp se consigue transfectando la línea celular humana 293T con plásmidos que codifican tres componentes: las proteínas nativas E1 y E2 de la envuelta de HCV, la cápsida y la polimerasa de un retrovirus y un genoma pro-viral que contiene un gen reportero (GFP o luciferasa) pero carece de los genes que codifican la polimerasa y las proteínas estructurales retrovirales. Mediante este sistema se generan partículas virales de un solo ciclo que permiten estudiar la entrada viral, desde la unión del virus a la célula hasta la liberación de la cápsida en el citoplasma.

Con el fin de estudiar la replicación viral surgieron los sistemas de replicones de HCV (Fig. 4). La primera generación de replicones (131) se generó a partir del clonaje de un genoma de HCV de genotipo 1b donde la región correspondiente a las proteínas estructurales está sustituida por un gen de resistencia a neomicina. Además, la inserción de un IRES permite la traducción de la región que codifica las proteínas no estructurales de HCV, que son las que controlan la replicación viral (82). El RNA sintético derivado de estas construcciones se introduce mediante electroporación en células Huh7, una línea derivada de un hepatoma humano, y su autorreplicación hace posible la selección de clones que son resistentes a neomicina y contienen gran cantidad de RNA y proteínas virales. Posteriormente se identificaron mutaciones de

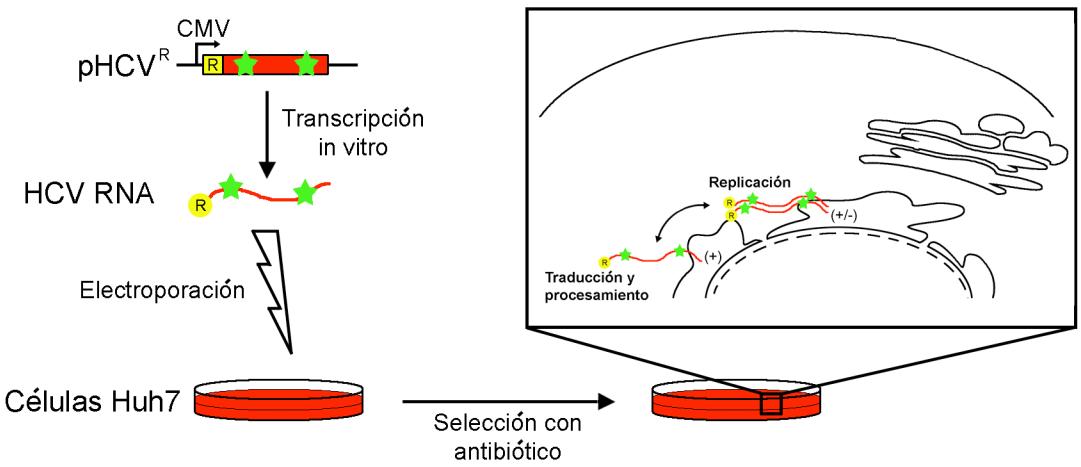


Figura 4. Sistema de replicones. Mediante transcripción *in vitro* se obtiene RNA que codifica la poliproteína viral y contiene un gen de resistencia a neomicina (R). Este RNA se introduce en las células mediante electroporación, y tras la selección con antibiótico se obtienen clones donde tiene lugar la replicación viral. Se cree que la presencia de mutaciones (estrellas verdes) necesarias para obtener una replicación robusta del RNA viral impide el ensamblaje de nuevos virus.

adaptación al cultivo celular que confieren al RNA viral una mayor capacidad de replicación, lo que hizo posible establecer replicones genómicos que expresan la poliproteína completa de HCV (171). El sistema de replicones constituye una herramienta muy útil para estudiar la replicación de HCV, la expresión y localización subcelular de las proteínas virales y su influencia en la célula huésped (82). Además, permite el desarrollo y evaluación de drogas antivirales dirigidas contra el proceso replicativo. Sin embargo, este sistema no produce virus infectivos (171), lo que lo limita para el estudio del ciclo viral completo. Finalmente, en 2005 se consiguió producir partículas virales infectivas generadas en cultivos celulares (HCVcc) a partir de un replicón derivado de la cepa JFH-1 de genotipo 2a, clonada a partir del suero de un

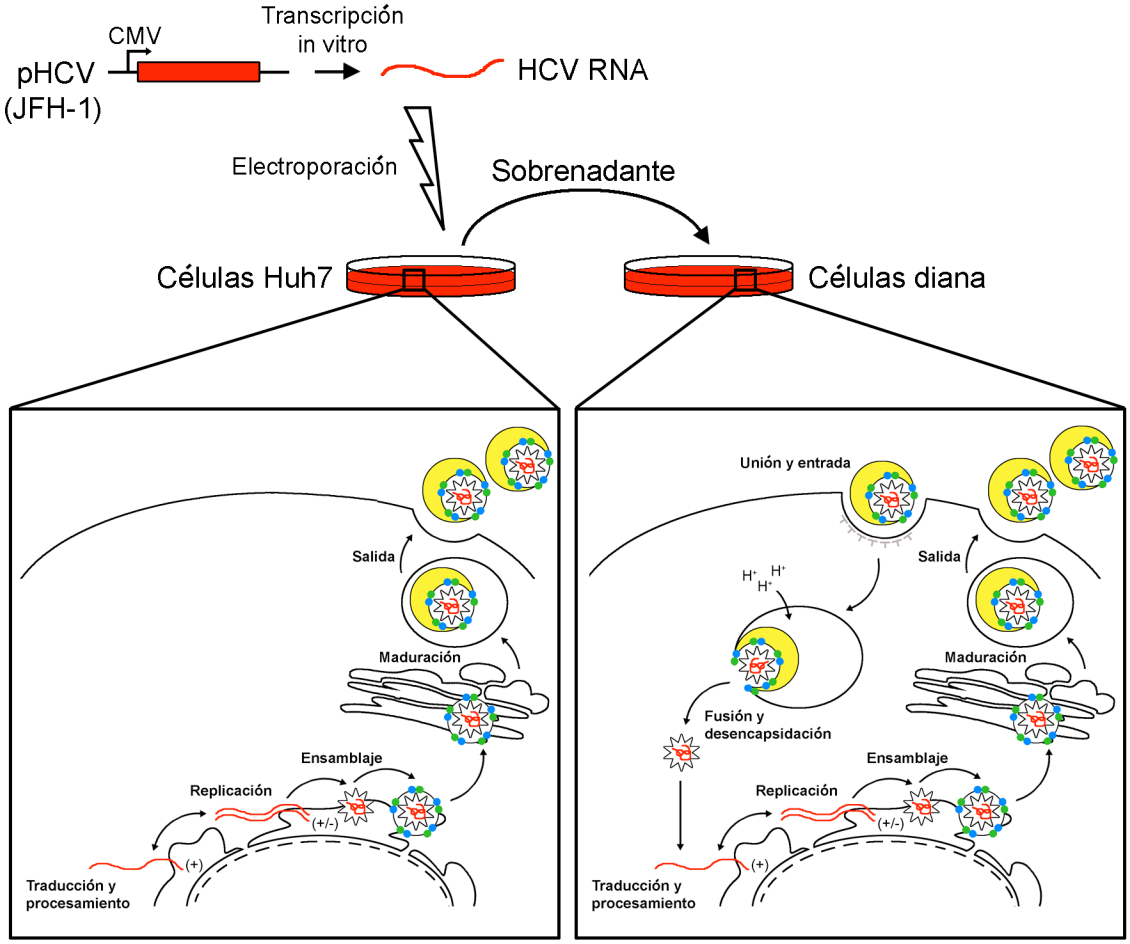


Figura 5. Sistema de HCVcc. Es un procedimiento idéntico al del sistema de replicones, solo que en este caso el aislado empleado (JFH-1) tiene la capacidad de replicarse de forma muy eficiente sin necesidad de la presencia de mutaciones adaptativas, lo que posibilita la generación de nuevas partículas virales infectivas.

paciente con hepatitis C fulminante (127, 216, 229) (Fig. 5). Posteriormente, a partir de este aislado se generaron quimeras intergenotípicas de HCVcc que contienen las proteínas estructurales, p7 y NS2 de cada uno de los genotipos principales (86, 87, 102, 191). El uso de HCVcc permite analizar la entrada, la replicación, el ensamblaje y la salida de los nuevos viriones de la célula infectada, siendo así válido para el estudio del ciclo viral completo.

2. LAS UNIONES INTERCELULARES ESTRECHAS O "TIGHT JUNCTIONS"

2.1 Estructura y función: mantenimiento de la polaridad celular

Las uniones intercelulares estrechas o "tight junctions" (TJs) son áreas de contacto entre células adyacentes donde el espacio intercelular queda ocluido por una red de fibras integrada en la membrana plasmática de ambas células (70). Las TJs, presentes fundamentalmente en células epiteliales y endoteliales (70), constituyen una pieza clave del complejo de adhesión intercelular epitelial, estando situadas en la zona más apical de dicho complejo por encima de los desmosomas y las uniones adherentes (13). Estas estructuras forman una barrera paracelular selectiva que restringe el paso de solutos a través de la monocapa celular, siendo ésta de crucial importancia para mantener separados diferentes compartimentos dentro del organismo (83). Además, mantienen la polaridad celular, ya que impiden la libre difusión de lípidos y proteínas entre los dominios basolateral y apical de la membrana plasmática (83).

A nivel molecular, las TJs son complejos multiproteicos formados por proteínas integrales de membrana que forman contactos homotípicos y heterotípicos con las células adyacentes e interaccionan mediante sus dominios intracelulares con proteínas citoplásmicas que sirven de anclaje al citoesqueleto de actina (3, 142). Las proteínas integrales de membrana se pueden clasificar según el número de dominios transmembrana que posean en a) proteínas con cuatro dominios transmembrana como ocludina, tricelulina, marvelD3 y la familia de las claudinas (13, 179, 200), y b) proteínas de la familia de las inmunoglobulinas con un solo dominio transmembrana como las moléculas de adhesión intercelular (JAMs), el receptor de Coxsackievirus y adenovirus (CAR) y la molécula de adhesión específica de células endoteliales (ESAM) (13, 70). Por otra parte, se ha descrito un gran número de proteínas citoplásmicas asociadas a TJs, entre las que se encuentran las proteínas zonula occludens (ZO)-1, ZO-2 y ZO-3. Muchas de estas proteínas adaptadoras interaccionan

además entre sí, lo que sugiere la existencia de un complicado entramado macromolecular vinculado a las TJs (3). Globalmente, todas estas interacciones regulan las funciones de la TJ y participan en la transducción de señales implicadas en el control de funciones celulares esenciales como la proliferación y diferenciación (3, 142).

La mayoría de las células epiteliales presentan una polaridad denominada “simple”. La membrana plasmática de estas células posee un dominio apical, localizado en el ápex celular y orientado hacia el lumen del órgano del que forman parte, y un dominio basolateral que corresponde al resto de la membrana (50) (Fig. 6). Por el contrario, los hepatocitos tienen varios dominios apicales y basolaterales. Los polos apicales de hepatocitos adyacentes forman una red continua de canalículos biliares en los cuales la bilis es secretada, mientras que los dominios basolaterales están en contacto con la sangre sinusoidal (50). El correcto funcionamiento del hígado depende de este fenotipo altamente polarizado, que a su vez es propiciado por las

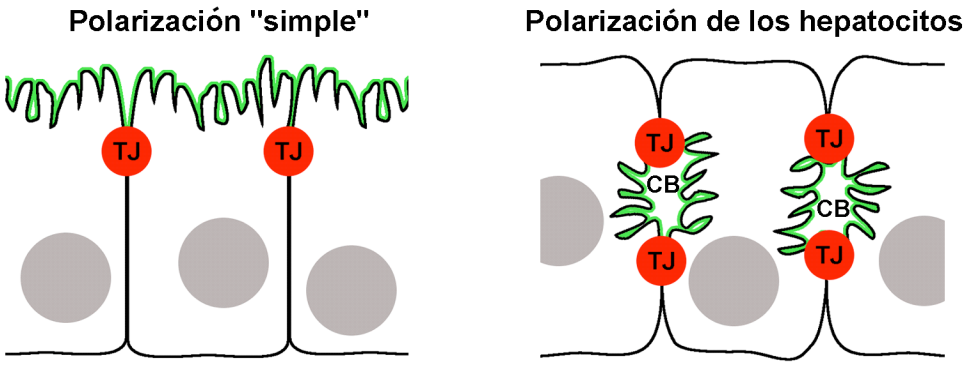


Figura 6. Esquema comparativo de la polarización epitelial “simple” y la polarización de los hepatocitos. Las TJs (rojo) constituyen una barrera entre el dominio apical (verde) y el dominio basolateral de la membrana plasmática. CB, canalículo biliar.

TJs. En el hígado, las TJs de los hepatocitos constituyen una barrera intercelular entre la sangre y la bilis. Además, permiten una distribución polarizada en membrana de los diferentes transportadores, lo que es esencial para el desarrollo de la función biliar (50). De hecho, se ha observado que la mayoría de enfermedades que afectan la secreción biliar llevan asociada la presencia de hepatocitos en los que la función de

las TJs está alterada (8, 189, 206). Por otra parte, se ha establecido una relación entre el desensamblaje de las TJs y el establecimiento y progresión del hepatocarcinoma (112, 163, 164, 192).

2.2 TJs como vías de entrada de virus

Las TJs sellan el epitelio formando una barrera entre el lumen y las capas internas de los órganos, que quedan así separados del medio externo. Por ello, las TJs constituyen una primera línea de defensa que impide la entrada de la mayoría de los microbios al interior del organismo. Sin embargo, algunas bacterias y virus han desarrollado estrategias específicas para modificar estas estructuras con el objetivo de penetrar en el huésped (84, 90). Así, se ha descrito que los rotavirus acceden al organismo desde el lumen del intestino mediante un mecanismo por el cual son capaces de alterar las TJs y abrir la barrera paracelular entre las células intestinales, quedando expuestas así las integrinas del dominio basolateral que les sirven de receptores (161).

Tanto la apariencia morfológica como la composición bioquímica de los complejos multiméricos que configuran las TJs han hecho que estas uniones intercelulares se hayan considerado durante mucho tiempo elementos rígidos y estáticos. Resulta difícil imaginar que una estructura con esas características pueda funcionar como receptor viral. Sin embargo, trabajos recientes han puesto de manifiesto que las TJs son sistemas muy dinámicos a nivel estructural y molecular, lo que además es importante para el correcto desarrollo de sus funciones (195, 199). Se ha observado que las TJs constituyen áreas que presentan un elevado reciclaje de membrana y un gran número de procesos endocíticos, siendo un punto estratégico de localización de proteínas implicadas en tráfico vesicular y señalización intracelular (84, 111, 223). Por todo ello, es posible que, haciendo uso de alguno de los elementos de las TJs, los virus aprovechen estas funciones fisiológicas para posibilitar su entrada en la célula huésped. Por ejemplo, la proteína asociada a TJs CAR interacciona y actúa como receptor del Coxsackievirus, del virus de la enfermedad vesicular porcina (SVDV) y de varios adenovirus (84). Además, los reovirus y el calcivirus felino utilizan JAM-A como receptor celular (19, 137). Por último, se ha demostrado que ocludina (26, 130, 173) (ver Anexo 2) y varios miembros de la familia de las claudinas (59, 149, 228) son co-receptores de HCV.

2.3 Proteínas asociadas a TJs implicadas en la infección por HCV

2.3.1 Claudina-1

Claudina-1 contiene cuatro dominios transmembrana y los extremos N y C-terminales intracelulares, y forma parte estructural y funcional de las TJs (3). Su identificación como co-receptor de HCV fue posible mediante la expresión de una biblioteca de cDNAs en células no permisivas para la infección, donde se observó que la expresión de claudina-1 les confería susceptibilidad a la infección (59). Posteriormente se descubrió que también claudina-6 y claudina-9 pueden funcionar como co-receptores de HCV (149, 228).

Al contrario que CD81 y SR-BI, hasta la fecha no se ha demostrado la existencia de una interacción directa entre claudina-1 y HCV (59, 116). Sin embargo, se ha descrito que dos aminoácidos presentes en el primer dominio extracelular de claudina-1 son indispensables para su función como co-receptor (59), sugiriendo la existencia de algún tipo de unión extracelular con el virus. Además, se ha observado la interacción entre claudina-1 y las proteínas de envuelta E1 y E2 cuando todas ellas son sobreexpresadas en líneas celulares (222). En estas condiciones, también se ha observado la interacción entre claudina-1 y CD81 tanto en la membrana plasmática como en vesículas intracelulares que expresan marcadores de endosomas tempranos (222), lo que sugiere una posible endocitosis conjunta de ambas proteínas. La interacción CD81-claudina-1 ha sido confirmada en varios sistemas experimentales (92, 93, 115, 148), y la ruptura de dicha interacción mediante anticuerpos bloqueantes o mutagénesis dirigida impide la unión eficaz del virus a la superficie celular, inhibiendo así la infección por HCV (92, 116). Además, estudios cinéticos empleando anticuerpos bloqueantes han demostrado que claudina-1 media una fase de la entrada de HCV íntimamente ligada a CD81 (116), reforzando la idea de que ambas proteínas pueden actuar conjuntamente en dicho proceso. La asociación entre claudina-1 y CD81 ocurre fundamentalmente en el dominio basolateral de la membrana plasmática (92), por lo que se ha sugerido que la entrada del virus tiene lugar fuera de la zona correspondiente a las TJs, haciendo uso de moléculas de claudina-1 que no forman parte de las uniones intercelulares. Esta hipótesis se apoya también en el hecho de que la entrada de partículas virales fluorescentes en células diana no esta asociada a zonas de contacto intercelular (41). Por otra parte, un mutante de claudina-1 que carece de su extremo C-terminal citoplasmático, necesario para su localización en las TJs (209), es capaz de actuar como co-receptor de HCV (59). Todo ello apunta a que

la función de claudina-1 en la entrada del virus está estrechamente relacionada con la de CD81, y que tiene lugar en zonas de la membrana que no forman parte de las TJs.

2.3.2 Ocludina

Ocludina, la primera proteína transmembrana asociada a TJs que se identificó (72), consta de cuatro dominios transmembrana y dos extracelulares, encontrándose sus extremos N y C-terminales en el citosol (3). Los primeros estudios descriptivos demostraron la presencia de ocludina en las fibras que constituyen las TJs (69, 74). Posteriormente, se demostró que las funciones de las TJs podían ser alteradas mediante la sobre-expresión de mutantes de ocludina o la administración de péptidos sintéticos correspondientes a sus dominios extracelulares (14, 15, 117, 144, 145, 210, 211, 221), poniendo de manifiesto la importancia de ocludina en las TJs. Sin embargo, su función no está claramente definida ya que se ha observado que los ratones knockout de ocludina, a pesar de presentar un fenotipo complejo con defectos en la reproducción y la diferenciación epitelial, poseen TJs bien desarrolladas (188, 193). Se han descrito varias isoformas de ocludina generadas por splicing alternativo, aunque se desconoce su función (79, 138, 160).

Se ha descrito que ocludina oligomeriza durante su trayecto hacia las TJs (49, 141), y que éste ocurre tras un paso previo de ocludina por la membrana basolateral de la célula dependiente del dominio C-terminal de la proteína (141). Asimismo, se ha observado que mutantes de ocludina que carecen de su segundo dominio extracelular se acumulan en la membrana basolateral, sugiriendo que dicho dominio tiene un papel importante en el ensamblaje estable de ocludina en las TJs (145). La localización y función de ocludina están controladas a diferentes niveles, incluyendo la participación de GTPasas, proteasas y citoquinas, interacciones con otras proteínas y modificaciones post-traduccionales como su fosforilación (62). Por ejemplo, el anclaje de ocludina al citoesqueleto de actina ocurre mediante la interacción directa de su dominio C-terminal con ZO-1, que a su vez interacciona con F-actina (61, 73). La internalización de ocludina se ha podido observar tanto *in vitro* como *in vivo*, y puede ocurrir en respuesta a diferentes estímulos (223). Además, dependiendo del estímulo, la internalización tiene lugar mediante macropinocitosis o endocitosis mediada por clatrina o caveolina (223). Una vez internalizada, ocludina puede reciclarse a membrana mediante un proceso dependiente de Rab13 (157). Por otra parte, la ubiquitín ligasa Itch puede marcar ocludina para su degradación mediante un mecanismo controlado por fosforilación (134, 159, 207).

La relación existente entre ocludina y la infección por HCV ha sido objeto de estudio de esta tesis doctoral (ver Anexos 1-4 y *Discusión*).

OBJETIVOS

OBJETIVOS

1) Estudiar la posible alteración de las uniones intercelulares estrechas inducida por HCV.

2) Estudiar el posible papel de ocludina, proteína asociada a uniones intercelulares estrechas, como co-receptor de HCV.

3) Desarrollar cultivos celulares tridimensionales altamente polarizados que permitan el estudio del ciclo completo de HCV.

ANEXO 1

ANEXO 1

Los componentes de la envuelta de HCV alteran la localización de las proteínas asociadas a TJs e inducen la retención de ocludina en el retículo endoplásmico.
Hepatology 2008; 48:1044-53.

En los hepatocitos, las TJs desempeñan un papel fundamental para el correcto desarrollo de las funciones hepáticas esenciales, incluyendo la formación y secreción de la bilis. Se ha observado que la colestasis, afección caracterizada por la disminución o interrupción del flujo de bilis, está asociada a la alteración funcional de las TJs. Durante la recidiva en la infección que ocurre en los pacientes infectados por HCV tras transplante hepático, se pueden alcanzar cargas virales en suero muy superiores a los valores anteriores al transplante. La reinfección conlleva en algunos casos la progresión de una forma colestática de hepatitis que conduce al fallo del injerto. Por ello, se decidió estudiar la posible influencia de las proteínas de HCV y la replicación viral sobre la integridad de las TJs.

Se observó que en células Huh7, tanto el replicón completo de HCV como la expresión transitoria de sus proteínas estructurales inducían la deslocalización de las proteínas asociadas a TJs ocludina, claudina-1 y ZO-1 de su posición normal entre células adyacentes. Este efecto no se detectó al emplear un replicón subgenómico de HCV en el que sólo se expresan las proteínas no estructurales del virus. Además, se comprobó que el replicón completo de HCV inducía la acumulación de ocludina en el retículo endoplásmico y la alteración de las funciones características de las TJs. Estos efectos se pudieron revertir en gran medida al eliminar el replicón viral mediante tratamiento con interferón alfa o con shRNA específico para HCV. Por otra parte, ensayos de inmunofluorescencia y análisis confocal sugirieron la existencia de una asociación entre los acúmulos intracelulares de ocludina y la proteína E2 de la envuelta viral, que fue confirmada mediante experimentos de co-inmunoprecipitación y “pull-down”. Por último, se pudo observar que la infección de células Huh7 con HCVcc también inducía la acumulación intracelular de ocludina. Estos datos parecen indicar que las proteínas estructurales de HCV, probablemente las de la envuelta viral, pueden provocar la alteración de las TJs. Esto podría explicar el mecanismo a través del cual altas cargas virales, como las observadas en las recidivas en la infección por HCV postransplante hepático, pueden comprometer la integridad de las TJs y contribuir al desarrollo de hepatitis colestática en determinados pacientes.

Hepatitis C Virus Envelope Components Alter Localization of Hepatocyte Tight Junction–Associated Proteins and Promote Occludin Retention in the Endoplasmic Reticulum

Ignacio Benedicto,[1,2] Francisca Molina-Jiménez,[1,2] Olga Barreiro,[3] Alejandra Maldonado-Rodríguez,[1,2] Jesús Prieto,[2,4,6] Ricardo Moreno-Otero,[2,5] Rafael Aldabe,[2,6] Manuel López-Cabrera,[1,2,7]* and Pedro L. Majano[1,2]*

Hepatocyte tight junctions (TJ) play key roles in characteristic liver functions, including bile formation and secretion. Infection by hepatitis C virus (HCV) may cause alterations of the liver architecture and disruption of the bile duct, which ultimately can lead to cholestasis. Herein, we employed the HCV replicon system to analyze the effect of HCV on TJ organization. TJ-associated proteins occludin, claudin-1, and Zonula Occludens protein-1 (ZO-1) disappeared from their normal localization at the border of adjacent cells in Huh7 clones harboring genomic but not subgenomic replicons expressing only the nonstructural proteins. Furthermore, cells containing genomic replicons showed a cytoplasmic accumulation of occludin in the endoplasmic reticulum (ER). TJ-associated function, measured as FITC-dextran paracellular permeability, of genomic replicon-containing cells, was also altered. Interestingly, clearance of the HCV replicon by interferon-α (IFN-α) treatment and by short hairpin RNA (shRNA) significantly restored the localization of TJ-associated proteins. Transient expression of all HCV structural proteins, but not core protein alone, altered the localization of TJ-associated proteins in Huh7 cells and in clones with subgenomic replicons. Confocal analysis showed that accumulation of occludin in the ER partially co-localized with HCV envelope glycoprotein E2. E2/occludin association was further confirmed by co-immunoprecipitation and pull-down assays. Additionally, using a cell culture model of HCV infection, we observed the cytoplasmic dot-like accumulation of occludin in infected Huh7 cells. *Conclusion:* We propose that HCV structural proteins, most likely those of the viral envelope, promote alterations of TJ-associated proteins, which may provide new insights for HCV-related pathogenesis. (HEPATOLOGY 2008;48:1044-1053.)

Abbreviations: ATPase, adenosine triphosphatase; ER, endoplasmic reticulum; GFP, green fluorescent protein; HCV, hepatitis C virus; IFN-α, interferon-alpha; shRNA, short hairpin RNA; STR, expression vector containing only the HCV structural proteins; TJ, tight junctions; ZO-1, Zonula Occludens protein-1;

From the [1]Molecular Biology Unit, Hospital Universitario de la Princesa, Madrid, Spain; [2]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Instituto de Salud Carlos III, Madrid, Spain; [3]Immunology Service, Hospital Universitario de la Princesa, Madrid, Spain; the [4]Liver Unit, Clínica Universitaria, Universidad de Navarra, Pamplona, Spain; the [5]Liver Unit, Hospital Universitario de la Princesa, Madrid, Spain; the [6]Division of Gene Therapy and Hepatology, Centro de Investigación en Medicina Aplicada, CIMA, Pamplona, Spain; and the [7]Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas (CSIC), Cantoblanco, Madrid, Spain.

**These authors contributed equally to this work.*

Received October 3, 2007; accepted June 2, 2008.

Supported in part by grants: (1) CIBERehd (R.M.-O. and M.L.-C.); (2) SAF2007-61201 (Ministerio de Educación y Ciencia) (M.L.-C.); (3) CP 03/0020 (Instituto Salud Carlos III); and (4) SAF2007-60667 (Ministerio de Educación y Ciencia) (P.L.M.). I.B. was financially supported by CIBERehd.

Address reprint requests to: Pedro Lorenzo Majano, Unidad Biología Molecular, Hospital Universitario de la Princesa, C/ Diego de León 62, 28006 Madrid, Spain. E-mail: pmajano.hlpr@salud.madrid.org; fax: (34)-91-3093911.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.22465

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Hepatitis C virus (HCV) infection is the most frequent cause of liver failure worldwide. More than 80% of acute infections become persistent, culminating in a chronic state that eventually progresses to liver fibrosis, cirrhosis, and hepatocellular carcinoma.[1] HCV is an enveloped positive-polarity RNA virus of the Flaviviridae family.[2] The life cycle of HCV includes its replication by a replisome, associated with altered membrane structures known as a "membranous web," and translation of the viral RNA to a polyprotein. This polyprotein is processed by host and viral proteases to yield 10 mature products, which include three structural proteins [the capsid protein (core) and two envelope glycoproteins (E1/E2)], the p7 protein, and the nonstructural proteins.[3] The development of autonomous subgenomic and genomic replication systems, HCV replicons, has provided a powerful tool to study the HCV biology.[4] Recently, an infectious cell culture model based on the production of infective HCV particles by transfection of Huh7 cells with genomic RNA (JFH1 clone) has been developed.[5]

Tight junctions (TJ) are major components of cell–cell adhesion complexes that separate apical from basolateral membrane domains and maintain cell polarity by forming an intramembrane fence that restricts diffusion of lipids in the exoplasmic leaflet of the plasma membrane ("fence" function).[6] Additionally, TJs form a paracellular barrier, which is not absolute because it allows the selective passage of certain solutes ("barrier" function). TJ multiprotein complex is composed of integral membrane proteins including occludin, which associate with actin cytoskeleton–interacting proteins such as zonula occludens protein-1 (ZO-1).[6] TJs constitute the major apical junctional structures among hepatocytes that exert a "barrier" function for the bile canalicular compartment and separate basolateral from canalicular membrane domains.[7] Bile secretion essentially requires TJs because they strictly seal the bile canaliculi, keeping bile away from the blood circulation.[8] In humans and animals, most cholestatic liver diseases are associated with profound changes in the pericanalicular region. Cholestatic hepatocytes show alterations of TJ functions, resulting in a deteriorated "barrier" function.[9-11] Recurrence of HCV infection after liver orthotopic transplantation is commonly associated with a rapid progression of a cholestatic form of hepatitis and leads to accelerated graft failure and death.[12] On recurrence, serum concentrations of HCV RNA may reach 10 to 20 times pretransplantation values, and high HCV protein expression in the transplanted liver is associated with the development of acute and chronic hepatitis. Considering that HCV infection is eventually associated, especially in patients with high viral load, with cholestasis[13] and that a relationship between cholestasis and TJ structure exists, we hypothesized that the HCV proteins may disrupt the intercellular adhesions affecting TJ. To explore this issue, we employed HCV subgenomic and genomic replicons and an infection system with HCV and analyzed TJ-associated protein organization.

Materials and Methods

Cell Culture, Generation of HCV Replicon-Containing Clones, and HCV Infection of Huh7 Cells. Huh7 cells and their derivatives were generated and grown as described.[14] Two genomic (HCV-G1 and HCV-G5) and two subgenomic (HCV-NS18 and HCV-NSA) replicon-containing clones were selected as representative of the 11 clones obtained (six genomic and five subgenomic) and used for further analysis. To eliminate the replicon, cells were either treated with human interferon-α_{2b} (IFN-α) or transduced with retrovirus encoding short hairpin RNAs (shRNAs). Huh7 cells were infected with HCV isolate JFH1,[5] and they were used 20 days postinfection when approximately 50% of cells were positive for HCV core protein, as determined by immunofluorescence.

Reverse Transcription Polymerase Chain Reaction. Total RNA was extracted and the complementary DNA was obtained from 1 μg RNA by reverse transcription. Quantitative polymerase chain reaction was carried out using two specific primer sets for HCV and histone H3.

Western Blots. Protein levels were analyzed with monoclonal antibodies anti-HCV core, anti-HCV E2, anti-HCV NS5A, and anti-p53, or polyclonal antibodies anti-occludin, anti-claudin-1, and anti–ZO-1.

Immunofluorescence Analysis and Confocal Microscopy. Cells were grown and processed as described in the supplementary materials and methods section.

Plasmid Constructs and Transfection. Transfections were performed using the expression vectors STR, pcDNA3.1Core1-191, Dyn2K44A-HA, and GFP (green fluorescent protein)-EPS15DIII. For co-culture experiments, Huh7 cells were transfected with pEGFP-C1 and processed as described in supplementary materials and methods. Plasmids coding for glutathione S-transferase (GST) and GST-Occludin were used in pull-down assays.

Co-immunoprecipitation and Pull-down Assays. Cleared lysates (1 mg protein) were subjected to immunoprecipitation with anti-E2, anti-core, anti-NS5A, or MOPC-21 mouse isotype control, and pull-down experiments employing GST or GST-occludin were performed.

Paracellular Permeability Assays. Cells were seeded onto Transwell filters, grown for the time indicated, and

processed as described in supplementary materials and methods.

Statistical Analysis. Results are expressed as the mean ± standard deviation. Statistical analysis was performed as described in supplementary materials and methods.

A detailed description of the protocols and reagents employed has been included in supplementary materials and methods.

Results

Disruption of TJ-Associated Protein Organization in Clones Harboring Genomic HCV Replicons. TJs separate apical from basolateral domains, maintaining cell polarity. To test the level of polarity ("fence" function) of Huh7 cells, we studied the localization of Na-K adenosine triphosphatase (ATPase), a protein excluded from the apical membrane in polarized cells, by immunofluorescence and confocal microscopy analysis. We observed that Na-K ATPase distribution was mostly limited to the lateral membranes, with no staining being observed along the apical domain of cells (Supplementary Fig. 1). As a control we included the polarized epithelial cell line Caco-2, which showed a Na-K ATPase localization very similar to Huh7 cells (Supplementary Fig. 1). Furthermore, we analyzed the localization of TJ-associated proteins, occludin and ZO-1, in Huh7 and Caco-2 cells by means of double-label immunofluorescence and confocal x-z section analysis. We observed that both proteins co-localized at the apical poles of lateral cell membranes, the typical distribution of occludin and ZO-1 in TJ-forming cells (Supplementary Fig. 1). Because TJs form a semipermeable paracellular diffusion barrier (that is crucial for epithelia and endothelia to separate different body compartments), we sought to test whether Huh7 monolayers presented "barrier" function by analyzing the dextran permeability with or without a previous calcium withdrawal (see Supplementary materials and methods). In these assays, calcium depletion led to a threefold increase of dextran permeability in Huh7 cells (Supplementary Fig. 1), thus confirming that these cells exhibited "barrier" function. In agreement with our results, a recent publication based on immunofluorescence and transepithelial electrical resistance analysis comparing Huh7 cells with cell lines that typically form (Caco-2) or do not form (HeLa) TJs strongly suggests that Huh7 cells possess at least some of the characteristics of TJ-forming cell types.[15]

To determine the effects of HCV on TJ-associated proteins, we analyzed the protein levels and subcellular localization of occludin, claudin-1, and ZO-1 in parental Huh7 and clones with either genomic or subgenomic HCV replicons previously characterized (Supplementary Fig. 2). Western blot analysis showed similar levels of these proteins in all cases (Fig. 1A). However, immunofluorescence analysis showed sharp differences between cell types. In Huh7 cells, occludin, claudin-1, and ZO-1 (Fig. 1B, a, f, and k, respectively) were localized at the borders between adjacent cells, indicating their intercellular junctional localization. In subgenomic clones, this localization was very similar to parental Huh7 cells (HCV-NS18, d, i, n; HCV-NSA, e, j, o). However, the staining pattern of TJ-associated proteins was completely different in genomic clones (HCV-G1, b, g, l; HCV-G5, c, h, m). Localization of occludin and claudin-1 nearly disappeared from intercellular junctions. Furthermore, ZO-1 immunostaining was confined to some scattered zones between adjacent cells (HCV-G1, l; HCV-G5, m). Interestingly, we observed a dot-like accumulation of occludin in the cytoplasm of the cells (HCV-G1, b; HCV-G5, c). This expression pattern of TJ-associated proteins was further confirmed in additional genomic and subgenomic clones (Supplementary Fig. 3), suggesting that the differences observed cannot be attributed to undefined clonal properties.

To analyze the possible alteration of the "barrier" function in HCV replicon clones, after growing cells on Transwell filters until confluence, we observed an increased paracellular permeability of HCV-G1 and HCV-G5 genomic clones when compared with parental Huh7 cells (Fig. 1C). Calcium-depleted HCV-G1 cells did not present a further increase in dextran permeability when compared with control HCV-G1 (Supplementary Fig. 1). Additionally, dextran permeability levels of calcium-depleted Huh7 cells were very similar to those of HCV-G1 cells (Supplementary Fig. 1). As a control for the calcium withdrawal effect, the polarized cell line Caco-2 was included. For unknown reasons, subgenomic clones grew as scattered cell groups and failed to form a complete monolayer; hence, these cells were not included in paracellular permeability assays. Furthermore, we studied the "fence" function by analyzing the localization of Na-K ATPase and ZO-1. Confocal analysis showed that Na-K ATPase was excluded from the apical poles of Huh7 and Caco-2 cells, whereas in HCV-G1 cells it appeared diffusely distributed across the entire plasmatic membrane (Supplementary Fig. 1). In contrast to Huh7 and Caco-2 cells, the TJ-associated protein ZO-1 did not localize at the apical poles of lateral cell membranes in HCV-G1 cells, suggesting that the TJ-associated "fence" function is altered in HCV-genomic clones

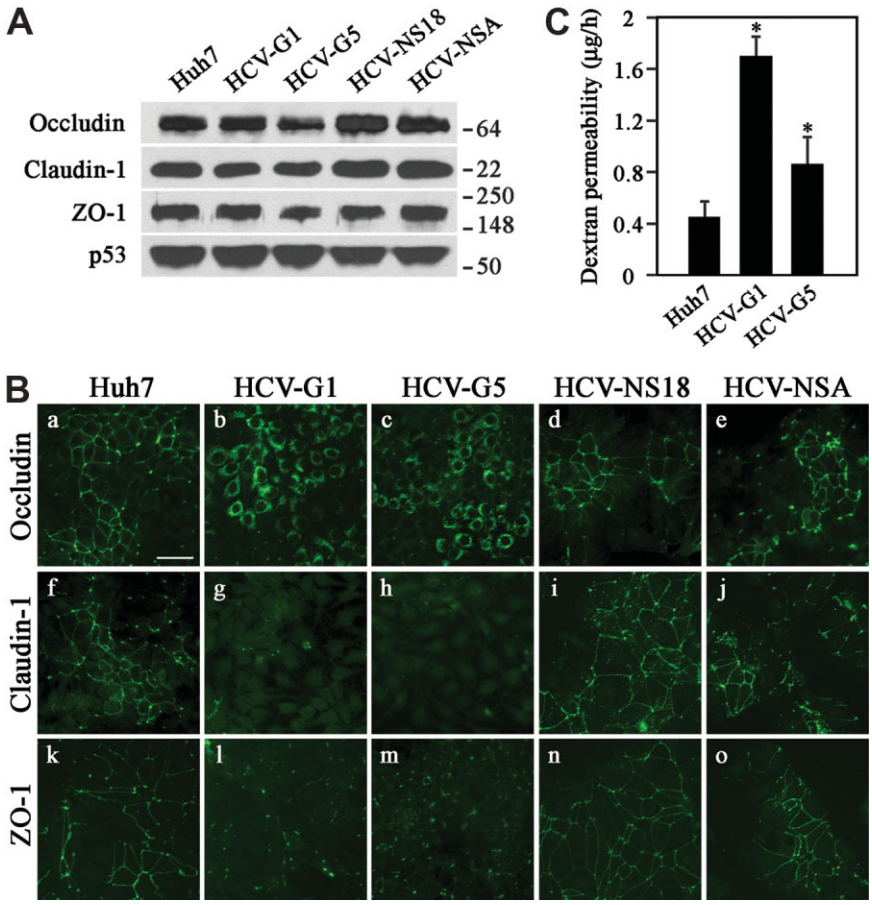


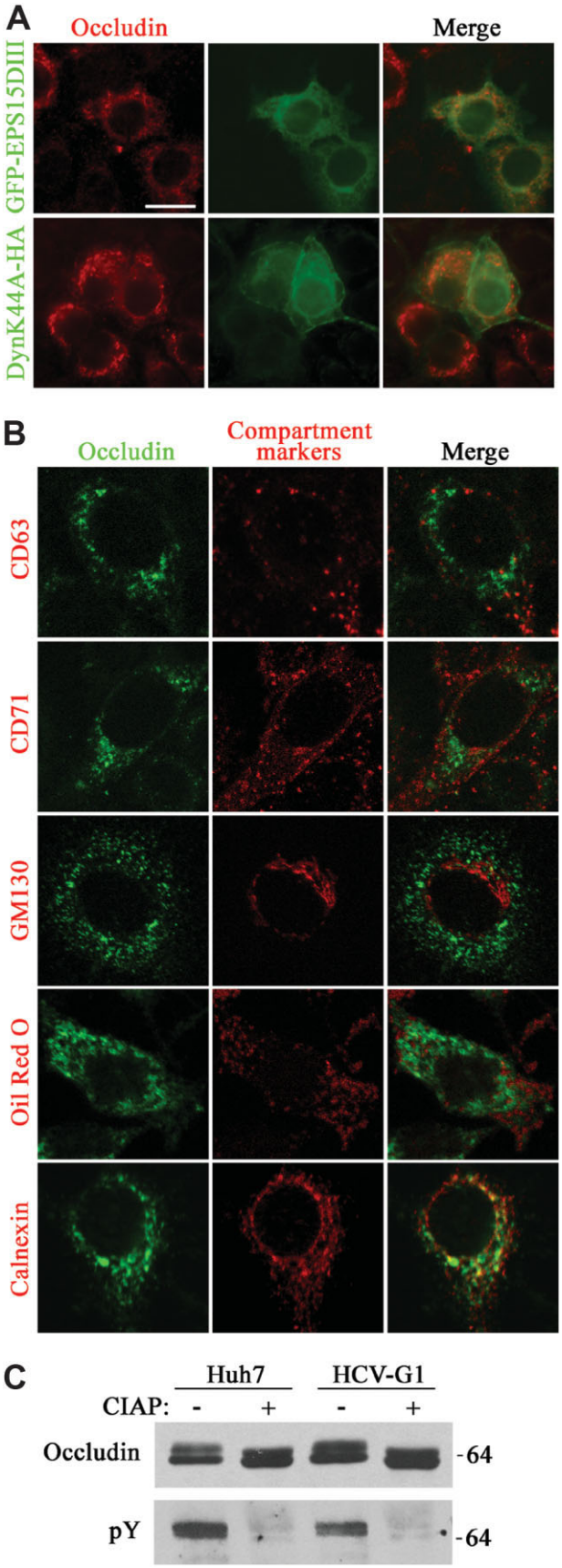
Fig. 1. Effects of HCV on TJ-associated proteins. (A) Occludin, claudin-1, and ZO-1 protein levels in Huh7 cells and genomic (HCV-G1/G5) or subgenomic (HCV-NS18/NSA) clones were analyzed by western blot. p53 levels were used as loading control. Molecular weight markers (kDa) are indicated on the right. Photographs are representative of five separate experiments. (B) Immunofluorescence analysis of TJ-associated proteins. Occludin (a-e), claudin-1 (f-j), and ZO-1 (k-o) localization in Huh7 cells, genomic and subgenomic HCV clones after 48 hours of cell culture. Bar, 50 μm. Results are representative of five separate experiments. (C) Paracellular permeability of Huh7 cells and genomic HCV replicon-containing clones. Cells were seeded onto polycarbonate membrane filters and maintained in culture for 3 days until confluence. The ability of fluorescein isothiocyanate–dextran to diffuse from the upper to the lower chamber of the Transwell was measured as described in supplementary materials and methods. Results are presented as the mean value $\pm$ standard deviation of six experiments (*$P < 0.05$ versus Huh7, Mann-Whitney U test).

(Supplementary Fig. 1). Taken together, all these results indicated that TJ-associated protein organization was altered in cells containing the complete HCV genome.

TJ-Associated Protein Occludin Is Retained in the Endoplasmic Reticulum by HCV. The results indicated that HCV structural proteins altered the subcellular localization of TJ-associated proteins, being especially striking in the unusual intracellular dot-like accumulation of occludin. Occludin internalization may be induced by several stimuli, including oxidative stress, proinflammatory cytokines, or growth factors.[16] To analyze whether internalization events could explain the cytoplasmic accumulation of occludin, transient transfection experiments using dominant negative constructs, which effectively alter clathrin (GFP-EPS15DIII) and clathrin/caveolin (Dyn2K44A-HA)-mediated internalization pathways[17] (Supplementary Fig. 4) were carried out. The data indicated that cytoplasmic occludin dot-like structures found in HCV genomic replicon cells were not affected after interfering with these pathways (Fig. 2A).

To define the intracellular localization of occludin in genomic replicon cells, confocal analysis of the colocalization of occludin with known markers of different cellular compartments was performed in HCV-G1 cells (Fig. 2B). These double-label immunofluorescence experiments showed that occludin did not colocalize with the Golgi complex (GM130) or lipid droplets (Oil Red O staining). In addition, no colocalization was observed with markers of the endocytic pathway, such as early endosomes (CD71) or late endosomes/lysosomes (CD63). Interestingly, there was a significant colocalization with the endoplasmic reticulum (ER) marker calnexin, which seemed to be co-distributed, thus indicating that occludin is mostly retained in ER structures. Given that the colocalization between occludin and the ER marker was only partial, we could not rule out the possibility that a proportion of occludin might be in another cellular compartment (for example, the ER-Golgi intermediate compartment).

Several reports have demonstrated that the distribution and function of occludin may be controlled by its phosphorylation state, suggesting that the levels of phospho-occludin may be a key determinant of the "barrier" properties of the TJ complex.[16] Thus, we sought to determine whether HCV-induced occludin mislocalization could be explained by an altered phosphorylation state of occludin. When protein extracts were more efficiently resolved by electrophoresis,



Western blot analysis revealed several occludin bands, similar in both Huh7 and HCV-G1 cells (Fig. 2C, top), which could represent posttranslational modifications or splice variants.[16] These bands migrated faster when lysates were incubated with alkaline phosphatase, suggesting the presence of phosphorylated forms of occludin. Efficient dephosphorylation was confirmed by reblotting the membrane using an anti-phosphotyrosine antibody (Fig. 2C, bottom). Data showed that the phosphooccludin pattern in HCV-G1 and Huh7 cells was similar, thus indicating that HCV triggered occludin dot-like accumulation in the ER without apparently affecting its phosphorylation state.

To rule out that the altered localization of occludin in genomic replicon-containing cells was attributable to a possible auto/paracrine effect of one or more secreted factor(s), co-culture experiments were performed. Green fluorescent protein (GFP)-expressing Huh7 cells were mixed with non-transfected Huh7 or HCV-G1 cells, and after 48 hours of culture the localization of occludin was analyzed by immunofluorescence (Supplementary Fig. 5). As expected, normal intercellular occludin staining was observed between transfected and nontransfected Huh7 cells (Supplementary Fig. 5, top). In the presence of HCV-G1 cells, occludin was also localized to the borders of adjacent transfected Huh7 cells (Supplementary Fig. 5, bottom), thus indicating that occludin mislocalization in genomic replicon-containing cells was not a response to soluble factors secreted by these clones.

Occludin Interacts With HCV E2 Glycoprotein. HCV replication takes place in a "membranous web," and most of the HCV proteins are located in membranous compartments.[18] To explore the possible association be-

Fig. 2. (A) Effect of interfering clathrin and clathrin/caveolin endocytic pathways on cytoplasmic dot-like accumulation of occludin. HCV-G1 cells were transiently transfected with plasmids harboring dominant negative constructs inhibiting clathrin (top, GFP-EPS15DIII) or clathrin/caveolin endocytic pathways (bottom, Dyn2K44A-HA) and after 48 hours of culture occludin localization was analyzed by immunofluorescence (red). EPS15DIII was detected coupled to GFP and Dyn2K44A-HA using an antibody against the hemagglutinin epitope (green). Bar, 20 μm. (B) Confocal immunofluorescence analysis of the intracellular distribution of occludin and cellular compartments. HCV-G1 cells grown on coverslips for 48 hours were fixed and processed for double-label immunofluorescence for occludin (green) and the following subcellular markers (red): CD71 (transferrin receptor, early endosomes), CD63 (late endosomes/lysosomes), GM130 (a Golgi matrix protein), and calnexin (an ER chaperone). Lipid droplets were stained with oil red O. Representative confocal images of individual cells are shown with the merged images on the right column. (C) Analysis of the phosphorylation state of occludin. Huh7 and HCV-G1 cells were lysed and treated with calf intestine alkaline phosphatase (CIAP) as described in supplementary materials and methods. Occludin migration was analyzed by western blot (top). Incubation with an anti-phosphotyrosine antibody (pY) was used as a positive control for phosphatase activity (bottom).

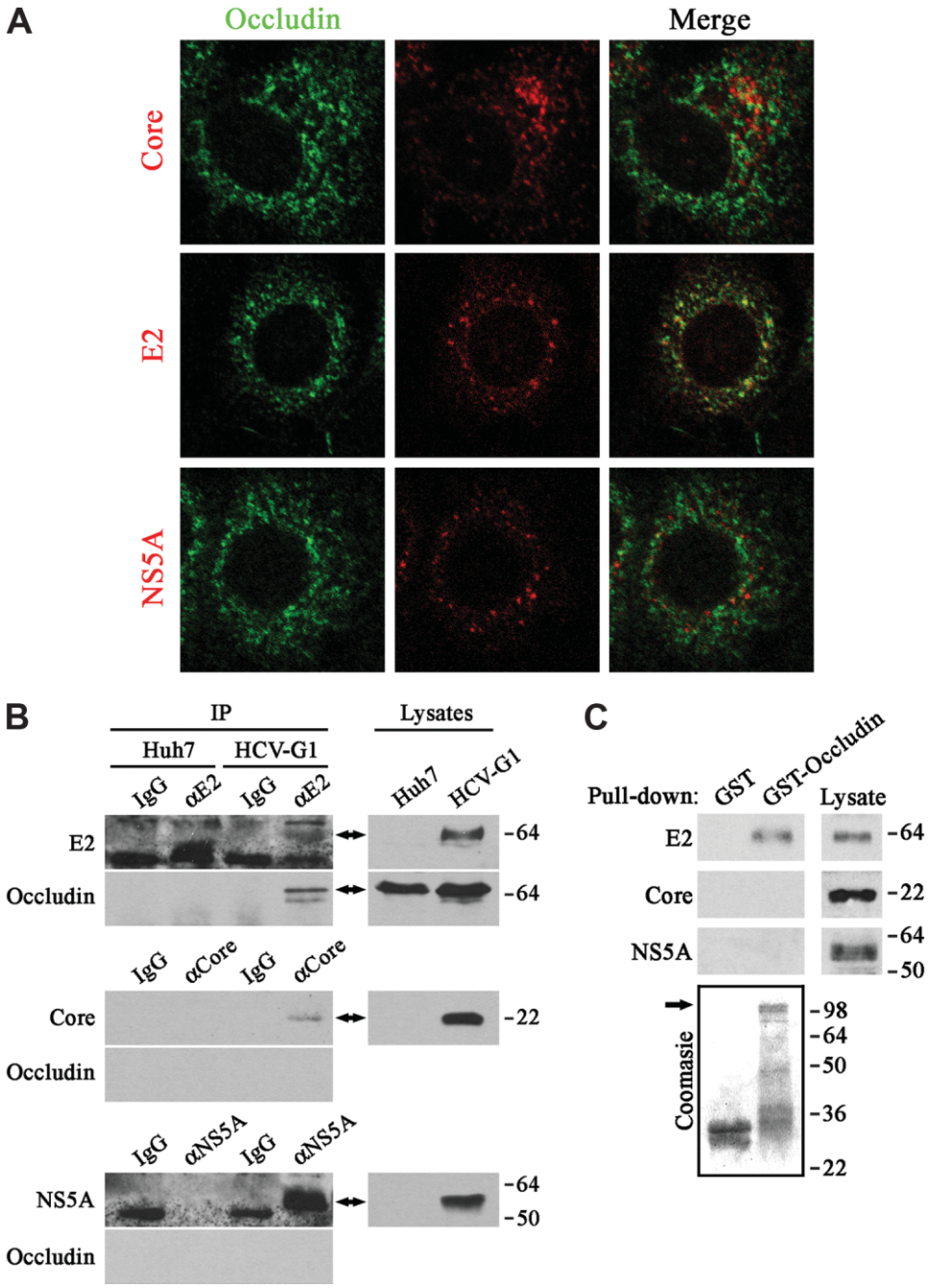


Fig. 3. Analysis of occludin/E2 association. (A) Confocal double-label immunofluorescence analysis of the intracellular distribution of occludin and HCV proteins. HCV-G1 cells were grown on coverslips, fixed, and processed for double-label immunofluorescence for occludin (green) and the HCV structural (core or E2) or nonstructural (NS5A) proteins (red). Reprsentative confocal images of individual cells are shown. (B) Lysates from Huh7 or HCV-G1 cells were immunoprecipitated using anti-E2, anti-core, or anti-NS5A monoclonal antibodies, or isotype-matched mouse immunoglobulins (IgG) as control. Western blot analysis of immunoprecipitates was performed with anti-E2, anti-core, or anti-NS5A (IP, upper panels) or anti-occludin (IP, lower panels) antibodies. Arrows indicate specific bands. (C) Pull-down assay with GST or GST-occludin and HCV-G1 cellular lysate. Bound proteins were subjected to western blot analysis using anti-E2, anti-core, or anti-NS5A antibodies (top). Coomassie brilliant blue staining of 1/10 of the GST proteins used is shown (bottom). Arrow indicates full-length GST-occludin. Molecular weight markers (kDa) are indicated on the right. Results are representative of three independent experiments.

tween occludin and viral proteins in genomic replicons, we performed confocal double-label immunofluorescence in HCV-G1 cells. Overall, E2 significantly co-localized with occludin in dot-like structures (Fig. 3A). In contrast, co-localization between occludin and NS5A or core was barely observed.

To further confirm the association between HCV E2 and occludin, co-immunoprecipitation assays using antibodies against E2, core, and NS5A were performed. In HCV-G1 lysates, occludin co-immunoprecipitated with E2 but not with core or NS5A (Fig. 3B). As expected, no signal was obtained from parental Huh7 immunoprecipitates (Fig. 3B). Additionally, we performed GST-based pull-down assays that showed that the fusion protein GST-occludin, but not GST, interacted with endogenous E2 from a cellular lysate of HCV-G1 cells (Fig. 3C). The specificity of this interaction was demonstrated because neither GST nor GST-occludin interacted with core or NS5A. Altogether, these data strongly suggested that E2 glycoprotein interacted with occludin, and that this interaction could be mediating occludin mislocalization.

Structural HCV Proteins Trigger Mislocalization of TJ-Associated Proteins. Genomic HCV clones, in

contrast to parental cells or subgenomic clones, showed an altered localization of TJ-associated proteins. These data suggested that HCV structural proteins could be responsible for the effects observed. To explore this possibility, we generated an expression vector (STR) containing only the HCV structural proteins. Western blot analysis showed that transient transfection of HCV-NS18 cells with STR resulted in the expression and correct processing of core and E2 proteins (Fig. 4, bottom right). Double-label immunofluorescence experiments carried out in HCV-NS18 cells showed that STR expression triggered the mislocalization of TJ-associated proteins, which was determined by the appearance of cytoplasmic dot-like accumulation of occludin and loss of delineated staining of ZO-1 at intercellular junctions (Fig. 4). Interestingly, E2 but not core showed co-staining with occludin. When HCV-NS18 cells were transfected with a plasmid coding only for the HCV core protein, no effects were observed (Fig. 4). Similar results were obtained using Huh7 cells (Supplementary Fig. 6), thus indicating that alterations of TJ-associated proteins induced by HCV structural proteins occurred independently of active viral replication. These data suggested that HCV glycoprotein E2 could be a determinant for the alteration of TJ-associated protein localization, although possible effects of E1 or p7 (also included in the STR construct) could not be ruled out.

Partial Recovery of TJ-Associated Protein Organization After Both IFN-α Treatment and HCV Silencing in Genomic Replicon Clones. We next examined whether the described alterations of TJ-associated proteins persisted after clearance of the HCV genomic replicon. As previously described,[19] prolonged exposure of the genomic replicon-containing HCV-G1 clone to IFN-α resulted in a total disappearance of the HCV replicon RNA (Fig. 5B) and viral proteins core and NS5A expression (Fig. 5A). The cells were considered as "cured" after 15 days of treatment. To avoid the possible interference of IFN-α with intercellular junction assembly or maintenance, the cured cells were grown for at least 1 week without the cytokine, before assessing their TJ-associated protein status. Immunofluorescence analysis showed a significant accumulation of occludin and ZO-1 at the border of adjacent cells in HCV-G1–cured cells when compared with untreated cells (Fig. 5G). Furthermore, a total disappearance of cytoplasmic dot-like accumulation of occludin was observed in these cells. In parental Huh7 cells, IFN-α treatment did not induce any changes. These findings suggest that the clearance of the replicon tends to reconstitute the localization of TJ-associated proteins. Similar results were obtained with genomic HCV-G5 cells (data not shown). Additionally, HCV-G1–cured cells showed a significantly decreased paracellular permeability when compared with untreated replicon-containing cells (Fig. 5C), thus suggesting that HCV elimination restores, at least partially, the epithelial sealing ability of HCV-G1 cells. Interestingly, all these data were further confirmed by silencing HCV using an shRNA retroviral transduction approach (Fig. 5D,E,F,H).

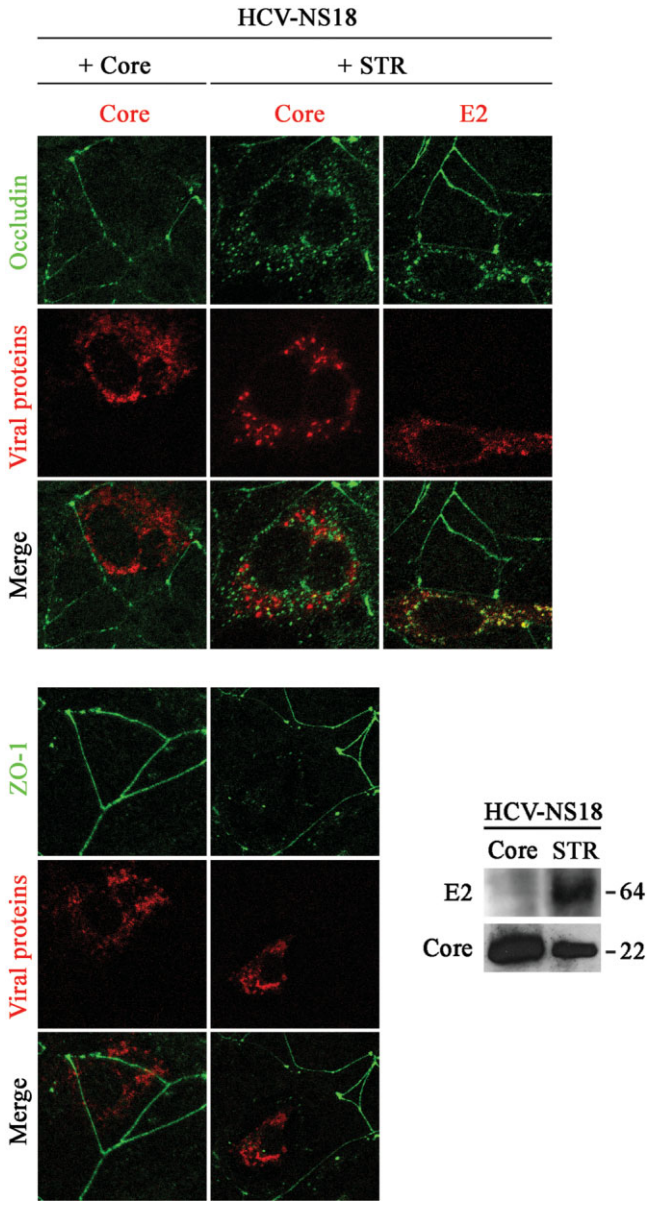


Fig. 4. Effect of HCV structural proteins on TJ-associated protein localization. HCV-NS18 cells were transfected with plasmids coding for HCV structural proteins (+ STR) or core protein (+ Core), and distribution of TJ-associated and viral proteins was analyzed by confocal immunofluorescence. Transfected HCV-NS18 cells were grown on coverslips for 48 hours, fixed, and processed for double-label immunofluorescence using anti-occludin or anti-ZO-1 (green) and anti-core or anti-E2 (red) antibodies. Representative confocal images of cells are shown. Core and E2 protein expression was confirmed by western blot (bottom right).

HCV Infection of Huh7 Cells Alters Occludin Distribution. Recently, a cell culture model has been devel-

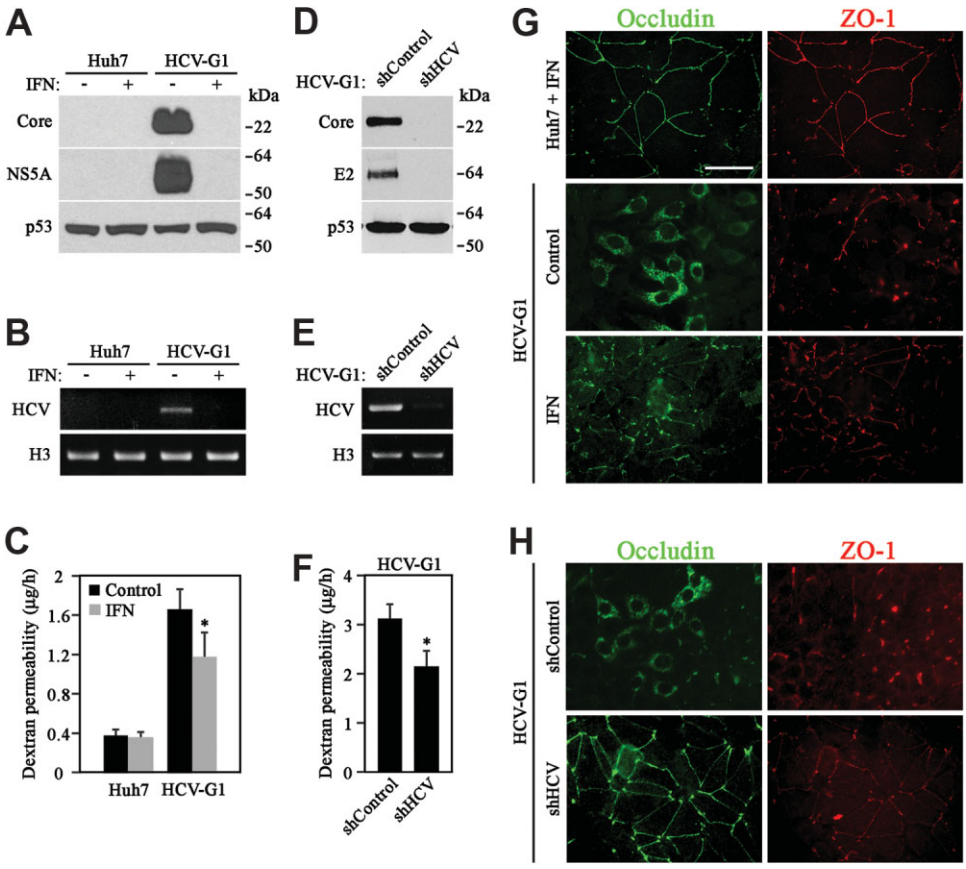


Fig. 5. Effects of HCV clearance on TJ-associated protein localization. Huh7 and HCV-G1 cells were cultured with human IFN-α (A, B, C, G), and HCV was silenced using shRNA technology on HCV-G1 cells (D, E, F, H) as described in supplementary Materials and Methods. (A, D) Core, NS5A, and E2 expression was analyzed by western blot. (B, E) HCV RNA levels were analyzed by quantitative reverse transcription polymerase chain reaction as described. To clearly show the obtained results, because there was no detectable HCV RNA in Huh7 and IFN-cured HCV-G1 cells, samples were electrophoresed in an agarose gel and stained with ethidium bromide. (C, F) Paracellular permeability was determined as described in supplementary Materials and Methods and Fig. 1 legend. Results are expressed as the mean value $\pm$ standard deviation of six experiments [$^*P < 0.05$, HCV-G1 cured cells (IFN) versus HCV-G1 control cells, and shHCV versus shControl, Paired t-test]. (G, H) Immunofluorescence analysis of occludin and ZO-1 localization. Bar, 50 μm. Results are representative of two independent experiments.

oped for HCV infection.[5] This allowed us to study the subcellular localization of occludin in the context of an infectious cycle. Huh7 cells were infected with HCV, and after 20 days of culture expression of core was analyzed by western blot (Fig. 6A), confirming that cells were successfully infected. We then examined occludin distribution in infected cells by double-label immunofluorescence for core and occludin (Fig. 6B). In core-expressing cells, occludin clearly disappeared from intercellular junctions and accumulated in a cytoplasmic dot-like fashion just resembling the pattern observed in genomic replicon-containing cells and STR-transfected Huh7 and HCV-NS18 cells.

Discussion

The most striking finding of this study is that HCV alters the localization of TJ-associated proteins. We show that HCV induces a loss of claudin-1, ZO-1, and occludin-delineated junctional accumulation with no changes in their expression levels. Additionally, the loss of TJ-associated protein organization is accompanied by a

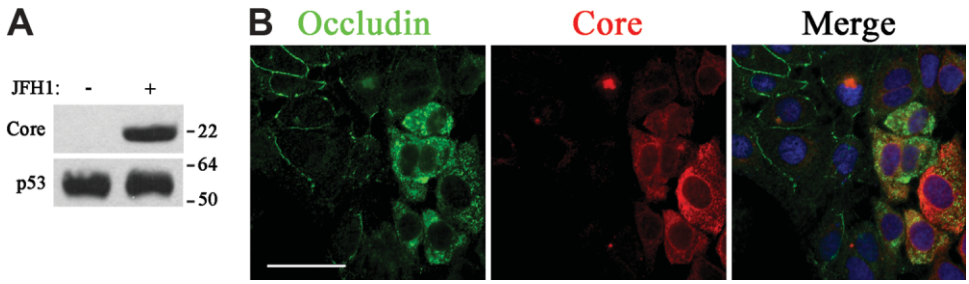


Fig. 6. HCV infection alters occludin localization. Cells were infected as described in Materials and Methods and cultured for 20 days. (A) Lysates of control or HCV-infected (JFH1) Huh7 cells were analyzed for core expression by western blot. (B) Double-label immunofluorescence analysis. Infected cells were processed for immunostaining using anti-core (red) and anti-occludin (green) specific antibodies. The merged image with the nuclei stained with 4′,6-diamidino-2-phenylindole (blue) is shown (right). Bar, 50 μm. Results are representative of two separate experiments.

marked intracellular dot-like accumulation of occludin in the ER, which was reproducible in three different systems: genomic HCV replicons, transient expression of structural HCV proteins, and HCV infection. TJs, the most apical intercellular junctions, form the rate-limiting barrier to water and solute flux through the intercellular cleft.[6] Our data demonstrate that clones with genomic HCV replicons present increased paracellular permeability when compared with parental Huh7 cells. Interestingly, additional support for the HCV-promoted alterations was provided by the assessment of TJ-associated protein disposition and "barrier" function after clearance of the HCV replicon. After HCV clearance by two different technical approaches, shRNA retroviral transduction or IFN-α treatment, genomic replicon cells exhibited a partial recovery of occludin and ZO-1 at the borders of adjacent cells and showed a significantly reduced paracellular permeability compared with control cells, which possibly reflects a more functional epithelial sealing. Furthermore, our experiments strongly suggest that the HCV structural proteins (most probably the envelope proteins), and not the nonstructural proteins or the viral replication process itself, are those that promote mislocalization of TJ-associated proteins as we demonstrated that: (1) subgenomic replicons that do not express core-E1-E2 proteins present well-localized occludin, ZO-1, and claudin-1 and (2) heterologous expression of core-E1-E2, but not core alone, in Huh7 cells or subgenomic clones alters localization of TJ-associated proteins. These data, supported by both occludin/E2 colocalization (confocal immunofluorescence assays) and interaction (co-immunoprecipitation/pull-down experiments), strongly suggest that E2 could play an important role in HCV-mediated mislocalization of TJ-associated proteins.

Different stimuli may disrupt TJ or prevent their assembly, including oxidative stress, inflammatory mediators, or growth factors.[6,20] Oxidative stress is considered to contribute to the pathogenesis of HCV-infected patients.[21] Additionally, the expression of either the entire polyprotein or the nonstructural proteins of HCV has been associated with the generation of reactive oxygen species.[22,23] Our experiments showed that localization of TJ-associated proteins was altered in cells harboring genomic but not subgenomic replicons. These data strongly suggest that the generation of a pro-oxidant cellular state is not sufficient to explain the observed HCV-mediated effects. Furthermore, our data indicate that other possible mechanisms, such as alteration of phosphorylation levels or internalization of occludin as well as the participation of soluble factors produced by HCV replicon-containing cells, are not likely to be involved in the HCV-promoted mislocalization of TJ-associated proteins.

Gap junctions and TJs play a central role in bile secretion.[8] In intrahepatic cholestasis, alterations of tight and gap junctional functions are frequently observed, which results in impaired intercellular communication and leaky canalicular structures.[9-11] Although the changes in gap junctions and TJs had been considered independent of each other, recent findings have shown that ZO-1 and occludin bind to connexins, which raises the possibility of either coordinate or reciprocal regulation of macromolecular complexes containing gap and TJ proteins.[24] The effects of HCV on gap junctions are currently being studied in our laboratory.

TJs have been classically considered as mere barriers. Recent findings, however, indicate that different types of signaling proteins and transduction pathways are associated with these intercellular structures.[25] They receive and convert intracellular signals to regulate junction assembly and function and transmit outside–inside signals to modulate gene expression and cell behavior. Taken together, these evidences indicate that fully formed TJs in high-density cell cultures might function as suppressors of signaling pathways that stimulate proliferation and inhibit differentiation.[20] Although changes in the architecture of TJs were described in cancerous tissues several decades ago, recent exciting results indicate that TJs may have a central role in these processes, directly correlating loss of TJs, cancer progression, and metastasis.[20] A major epidemiological association between HCV and hepatocellular carcinoma is evident; indeed, HCV proteins can modify intracellular signalling pathways associated with cell proliferation, differentiation, and apoptosis.[3] These facts suggest that a relationship between disruption of TJs and alteration of signal transduction induced by HCV could exist, but this possible association remains to be addressed.

Viral entry into the host cell requires the binding of proteins present at the surface of the virion to one or more molecule(s) on the cell surface. Viral attachment can occur as a multistep process, involving more than one type of receptors or co-receptors. Several cellular molecules have been identified as putative receptors for HCV, including tetraspanin CD81, scavenger receptor class B type I, and mannose-binding lectins dendritic cell-specific intracellular adhesion molecules (ICAM)-3 grabbing non-integrin (DC-SIGN) and liver/lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN). Several TJ-associated proteins such as Coxsackievirus and adenovirus receptor[26] and junctional adhesion molecule[27] act as virus receptors. Virus–Coxsackievirus and adenovirus receptor interactions are likely to be responsible for the observed

disruption of TJs on binding of adenovirus fibers to Coxsackievirus and adenovirus receptor, which results in apical escape of the virus after basolateral secretion.[26] Recently, Evans and colleagues[28] described that claudin-1 is implicated in late steps of HCV infection, and that this molecule could be considered as a necessary co-receptor for HCV infection. E2 glycoprotein, a component of HCV envelope, is the subunit involved in the interaction with most of the putative receptors. Although the data presented herein do not demonstrate whether a direct interaction E2/occludin occurs, our co-localization and co-immunoprecipitacion/pull-down experiments strongly suggest that this intracellular association may exist. This raises the exciting possibility that the E2/occludin association could also take place extracellularly, being implicated in the virus–host interaction. Further studies are needed to test this hypothesis. Very recently, Mee et al.[29] proposed a model in highly polarized cells in which TJs provide a physical barrier for viral access to cellular receptors. According to this model, HCV-mediated TJ disruption could probably be an advantage to the virus to facilitate subsequent viral entry or release.

In conclusion, we have demonstrated for the first time that HCV structural proteins induce alterations of TJ-associated protein organization, possibly via glycoprotein E2/occludin interaction and ER retention. We propose that this phenomenon may provide a mechanism to explain how HCV is implicated in development of cholestasis and probably in other cellular alterations induced by viral infection.

Acknowledgment: The authors thank Drs. Cosset, Bartenschlager, Wakita, del Pozo, Sánchez-Mateos and Sánchez-Madrid for providing us with critical reagents. The authors also thank Dr. Viton for cell sorting and Drs. Aguilera and de la Fuente for statistical analysis.

References

1. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. Lancet 2003; 362:2095-2100.
2. Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature 2005;436:933-938.
3. Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. HEPATOLOGY 2004;39:5-19.
4. Bartenschlager R. The hepatitis C virus replicon system: from basic research to clinical application. J Hepatol 2005;43:210-216.
5. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
6. Aijaz S, Balda MS, Matter K. Tight junctions: molecular architecture and function. Int Rev Cytol 2006;248:261-298.
7. Easter DW, Wade JB, Boyer JL. Structural integrity of hepatocyte tight junctions. J Cell Biol 1983;96:745-749.
8. Kojima T, Yamamoto T, Murata M, Chiba H, Kokai Y, Sawada N. Regulation of the blood-biliary barrier: interaction between gap and tight junctions in hepatocytes. Med Electron Microsc 2003;36:157-164.
9. Anderson JM. Leaky junctions and cholestasis: a tight correlation. Gastroenterology 1996;110:1662-1665.
10. Trauner M, Meier PJ, Boyer JL. Molecular pathogenesis of cholestasis. N Engl J Med 1998;339:1217-1227.
11. Sakisaka S, Kawaguchi T, Taniguchi E, Hanada S, Sasatomi K, Koga H, et al. Alterations in tight junctions differ between primary biliary cirrhosis and primary sclerosing cholangitis. HEPATOLOGY 2001;33:1460-1468.
12. Schluger LK, Sheiner PA, Thung SN, Lau JY, Min A, Wolf DC, et al. Severe recurrent cholestatic hepatitis C following orthotopic liver transplantation. HEPATOLOGY 1996;23:971-976.
13. Doughty AL, Spencer JD, Cossart YE, McCaughan GW. Cholestatic hepatitis after liver transplantation is associated with persistently high serum hepatitis C virus RNA levels. Liver Transpl Surg 1998;4:15-21.
14. Pietschmann T, Lohmann V, Kaul A, Krieger N, Rinck G, Rutter G, et al. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 2002;76:4008-4021.
15. Yang W, Qiu C, Biswas N, Jin J, Watkins SC, Montelaro RC, et al. Correlation of the tight junction-like distribution of claudin-1 to the cellular tropism of HCV. J Biol Chem 2008;283:8643-8653.
16. Feldman GJ, Mullin JM, Ryan MP. Occludin: structure, function and regulation. Adv Drug Delivery Res 2005;57:883-917.
17. del Pozo MA, Balasubramanian N, Alderson NB, Kiosses WB, Grande A, Anderson RG, et al. Phospho-caveolin-1 mediates integrin-regulated membrane domain internalization. Nat Cell Biol 2005;7:901-918.
18. Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, et al. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol 2002;76: 5974-5984.
19. Frese M, Pietschmann T, Moradpour D, Haller O, Bartenschlager R. Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. J Gen Virol 2001;82:723-733.
20. Gonzalez-Mariscal L, Lechuga S, Garay E. Role of tight junctions in cell proliferation and cancer. Prog Histochem Cytochem 2007;42:1-57.
21. Larrea E, Beloqui O, Munoz-Navas MA, Civeira MP, Prieto J. Superoxide dismutase in patients with chronic hepatitis C virus infection. Free Radic Biol Med 1998;24(7-8):1235-1241.
22. Piccoli C, Scrima R, Quarato G, D'Aprile A, Ripoli M, Lecce L, et al. Hepatitis C virus protein expression causes calcium-mediated mitochondrial bioenergetic dysfunction and nitro-oxidative stress. HEPATOLOGY 2007;46:58-65.
23. Waris G, Turkson J, Hassanein T, Siddiqui A. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. J Virol 2005;79:1569-1580.
24. Kojima T, Spray DC, Kokai Y, Chiba H, Mochizuki Y, Sawada N. Cx32 formation and/or Cx32-mediated intercellular communication induces expression and function of tight junctions in hepatocytic cell line. Exp Cell Res 2002;276:40-51.
25. Matter K, Balda MS. Epithelial tight junctions, gene expression and nucleo-junctional interplay. J Cell Sci 2007;120:1505-1511.
26. Walters RW, Freimuth P, Moninger TO, Ganske I, Zabner J, Welsh MJ. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 2002;110:789-799.
27. Barton ES, Forrest JC, Connolly JL, Chappell JD, Liu Y, Schnell FJ, et al. Junction adhesion molecule is a receptor for reovirus. Cell 2001;104:441-451.
28. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801-805.
29. Mee CJ, Grove J, Harris HJ, Hu K, Balfe P, McKeating JA. Effect of cell polarization on hepatitis C virus entry. J Virol 2008;82:461-470.

SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Cell culture, generation of HCV replicon-containing clones and HCV infection of Huh7 cells.

Caco-2, Huh7 cells and their derivatives were grown at 37ºC with a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2mM L-glutamine, 50 µg/ml gentamycin, 100 U/ml penicillin and 100 µg/ml streptomycin.

Huh7 cells expressing full-length or subgenomic genotype 1b (Con1, EMBL database accession number AJ238799) HCV replicons were established as previously described (14). Briefly, the constructs pI_{389}/Core-3'/5.1 and pI_{377}/NS3-3' were linearized with *Sca*I and used as templates for RNA synthesis using the T7 RNA polymerase (Promega, Madison, WI). 20 µg of synthesized RNA were used to electroporate 10^7 Huh7 cells and 24 hours later 500 µg/ml of G418 were added. Twice a week culture medium supplemented with G418 was replaced and 4 weeks after transfection the colonies resistant to G418 were isolated. Two genomic (HCV-G1 and HCV-G5) and two subgenomic (HCV-NS18 and HCV-NSA) replicon-containing clones were selected as representative of the eleven clones obtained (six genomic and five subgenomic) and used for further analysis. To eliminate the replicon, the cells were treated for 2 weeks with 500 IU/ml of human IFN-α_{2b} (PEG-Intron, Schering-Plough Corp., Kenilworth, NJ) in the absence of G418. The cured cells were maintained without G418. Alternatively, an shRNA approach was employed (see below).

Huh7 cells were infected with HCV isolate JFH1 (moi 0,01) (5) and used 20 days post-infection when about 50% of cells were positive for HCV core protein, as determined by immunofluorescence.

RT-PCR

Total RNA was extracted with TRI Reagent (Ambion Inc., Austin, TX), and the cDNA was obtained from 1 µg of RNA by reverse transcription. Quantitative PCR was carried out in a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using a SYBR Green kit (Roche Diagnostics GmbH) and two specific primer sets (5'-

CCTGTGAGGAACTACTGTCT-3' and 5'-CTATCAGGCAGTACCACAAG-3' for HCV, spanning 255 nucleotides of the 5' non-translated region; 5'-AAAGCCGCTCGCAAGAGTGCG-3' and 5'-ACTTGCCTCCTGCAAAGCAC-3' for histone H3). The number of HCV RNA copies was determined by crossing point interpolation into standard curves, which were generated by reverse transcription of serially diluted, *in vitro* synthesized viral RNA (genomic or subgenomic) followed by quantitative PCR. The total amount of RNA per reaction was kept constant (1 µg) by the addition of Huh7 RNA.

Western blots

After washing with phosphate-buffered saline (PBS), cells were lysed on the plate with 100 µl of Laemmli buffer and boiled for 5 minutes. Proteins were separated on a SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) that were incubated in blocking solution [5% skimmed milk in Tris-buffered saline (TBS)] for 1 hour at room temperature. Membranes were then incubated overnight at 4ºC with monoclonal antibodies (diluted in TBS, 0.1% Tween-20, 1% skimmed milk) anti-HCV core (clone C7-50, Affinity BioReagents, Goleen, CO), anti-HCV E2 (clone 4F6/2, Austral Biologicals, San Ramon, CA), anti-HCV NS5A (Virostat, Portland, ME) and anti-p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or polyclonal antibodies anti-occludin, anti-claudin-1 and anti-ZO-1 (Zymed, San Francisco, CA). Membranes were washed three times in TBS-T (0.1% Tween-20 in TBS) and incubated with a peroxidase-labeled goat anti-mouse or anti-rabbit IgG (Pierce, Rockford, IL) diluted in TBS-T for 45 minutes at room temperature. After three washes in TBS-T, membrane-bound antibody was visualized with the SuperSignal West Pico Chemiluminiscent Substrate (Pierce).

Immunofluorescence analysis and confocal microscopy

Cells were grown on coverslips for 48 hours or onto 6.5 mm diameter Transwell filters with a 0.4 µm pore size (Corning Inc., Corning, NY) for 72 hours or 10 days as indicated. Cells were washed with PBS and fixed in methanol for 20 minutes at -20ºC. Alternatively, cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature and permeabilized with 0.1% NP-40 in PBS for 10 minutes at room temperature, obtaining in both cases similar results. Cells were blocked with TNB [0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagent (Boehringer Mannheim GmbH, Mannheim, Germany)] for 30 minutes at 37ºC and incubated with the indicated

antibodies diluted in TNB for 1 hour at 37ºC. After washing with 0.1% NP-40 in PBS, cells were incubated with the secondary antibodies Alexa 488 or rhodamine X-conjugated goat anti-mouse or anti-rabbit (Molecular Probes, Inc., Eugene, OR) for 20 minutes at 37ºC. After washing, the coverslips or filters were either mounted on DakoCytomation Fluorescent Mounting Medium (DAKO A/S, Glostrup, Denmark), or for double stainings, samples were incubated sequentially with the indicated primary and secondary antibodies and mounted. A monoclonal anti-occludin antibody (Zymed) was used for double-label immunofluorescences when needed. The preparations were analyzed with a Leica TCS-SP (Leica Microsystems, Heidelberg, Germany) confocal microscope or a conventional Leica DMR photomicroscope equipped with QFISH software.

Monoclonal antibody anti-HA (12CA5) was purchased from Boehringer Mannheim GmbH. Monoclonal antibodies used to stain the different subcellular compartments were the following: anti-calnexin (BD Transduction Laboratories, Lexington, KY), anti-GM130 (BD Biosciences, Franklin Lakes, NJ), anti-CD63 (TEA 3/18) and anti-CD71 (FG 2/12) (kindly provided by Dr. Sanchez-Madrid). Oil Red O was purchased from Sigma (St. Louis, MO) and used as previously described (30).

Plasmid constructs and transfection

The expression vector STR was generated by cloning into *Eco*RI and *Xba*I sites of pcDNA3.1 (Invitrogen, Carlsbad, CA) a PCR product encoding the HCV structural proteins core, E1, E2, p7 and the first 33 aminoacids of the non-structural protein NS2. The PCR was carried out with the primers 5'-ACTGGAATTCACCATGGGCACGAATCCTA-3' (forward) and 5'-ACTGTCTAGAGCCTAGCGAGGAACAGC-3' (reverse), using cDNA from a pI_{389}/Core-3'/5.1 clone (HCV-G1) as template. The plasmid pcDNA3.1Core1-191, coding for full-length core from genotype 1b HCV, has been previously described (31).
The plasmid GST-Occludin was constructed by cloning into the *Eco*RI site of pGEX-2T (Amersham Biosciences, Buckinghamshire, UK) a PCR product coding for full-length human occludin. The constructs Dyn2K44A-HA (HA-tagged dominant-negative dynamin-2 mutant) and GFP-EPS15DIII (GFP-tagged carboxy-terminal domain of Eps15, a selective inhibitor of the classic clathrin pathway) were kindly provided by Dr. M. A. del Pozo (17). In order to test the functionality of these constructs in our system, clathrin-dependent endocytosis of the transferrin receptor in transfected cells was assessed. Cells were grown on coverslips and Texas Red-labelled transferrin

(Molecular Probes Inc.) was added to media (500 µg/ml). After 20 minutes at 37ºC cells were washed with PBS, fixed and either mounted (for GFP-EPS15DIII transfectants) or processed for immunofluorescence with anti-HA and Alexa 488-conjugated goat anti-mouse (for Dyn2K44A-HA transfectants). Transfections were performed with 2 µg of the indicated plasmids, employing Lipofectin (Invitrogen) according to manufacturer's instructions. Cells were fixed or lysed 48 hours after transfection.

For co-culture experiments, Huh7 cells were transfected with pEGFP-C1 (Clontech Laboratories, Inc., Mountain View, CA). After 24 hours of culture, GFP-expressing cells were purified by flow cytometry with a FACSAria cell sorter (BD Biosciences) and mixed with non-transfected Huh7 or HCV-G1 cells in a 1:4 ratio. Cells were seeded onto coverslips and fixed for immunofluorescence 48 hours later.

Alkaline phosphatase treatment

After 48 hours of culture, cells were washed with PBS and lysed in SDS lysis buffer (25 mM Tris-HCl pH 7.4, 4 mM EDTA, 1% SDS). The lysate was diluted 1:10 in dilution buffer (25 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 1% Triton X-100), sonicated for 2 × 20 seconds in a Soniprep 150 (MSE Ltd., Crawley, UK) and incubated in a final volume of 200 µl with or without 20 units of calf intestine alkaline phosphatase (CIAP) (Roche Diagnostics GmbH) for 1 hour at 37ºC. Samples were boiled in Laemmli buffer and analyzed by Western blot. Protein dephosphorylation was monitored by reblotting the membranes with anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Lake Placid, NY).

Co-immunoprecipitation

Huh7 and HCV-G1 cells were washed with ice-cold PBS and lysed in buffer containing 150mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP-40, 1mM NaF, 1mM Na_3VO_4 and EDTA-Free Halt Protease Inhibitor Cocktail (Pierce) for 30 minutes on ice. Following centrifugation (23,440 × *g*) at 4ºC for 20 minutes, the protein concentration of the supernatants was measured with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Cleared lysates (1 mg of protein) were then subjected to immunoprecipitation with 2 µg of anti-E2, anti-core, anti-NS5A or MOPC-21 mouse isotype control (Sigma) overnight at 4ºC. Protein G-Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was added and incubation continued for 1 hour at 4ºC. Precipitates were washed three times with ice-cold lysis buffer, resuspended in Laemmli buffer and boiled for 10

minutes. Bound proteins were separated on a SDS-polyacrylamide gel and analyzed by Western blotting using the indicated antibodies.

Purification of recombinant proteins and pull-down assays

GST and GST-Occludin were expressed in *E. coli*, purified with glutathione-Sepharose 4B (Amersham Biosciences) and incubated with HCV-G1 cellular extracts as previously described (31). Bound proteins were analyzed by Western blot using anti-E2, anti-core and anti-NS5A Abs.

HCV shRNA retroviral transfer

Complementary DNA oligonucleotides containing either the sequence corresponding to nucleotides 321-339 of the HCV Con1 sequence (32) or a sequence that does not pair with any human mRNA, were annealed and inserted between the *Bgl*II and *Hind*III sites of pSUPER.retro.puro (Oligoengine, Seattle, WA) according to the manufacturer's instructions to generate the constructs shHCV and shControl, respectively. 3×10^6 293T cells were seeded and 24h later transfected with the retroviral construct (10.5 µg), pNGVL-MLV-gag-pol (33) (12 µg) and pVSV-G (Clontech) (7.5 µg) by the calcium phosphate method. Media was changed 24h post-transfection and supernatants were filtered (0.45 µm pore) 24h and 48h later and used immediately for cell transduction. 10^5 HCV-G1 cells were seeded and 24h later diluted retroviral supernatants (1:2 in fresh medium) containing 6 µg/ml polybrene (Sigma) were added. Cells were incubated overnight before a second transduction, and after 24h cells were washed and fresh media was added. 3 days after the last transduction, shRNA-containing cells were selected with 4 µg/ml puromycin for 1 week and pooled before quantifying HCV RNA and protein levels. HCV-G1 shControl cells were mantained with G418 and puromycin, and HCV-G1 shHCV cells only with puromycin. No selection antibiotic was added to media during the course of experiments.

Paracellular permeability assays

2×10^4 cells were seeded onto 6.5 mm diameter Transwell filters with a 0.4 µm pore size (Corning Inc.) and grown for 72 hours. Caco-2 cells were cultured for 10 days as a model for a fully polarized cell line (29). Polarity of Huh7, HCV-G1 and Caco-2 cells in these conditions was evaluated by immunofluorescence (as described above)

employing an antibody against Na, K-ATPase (Abcam, Cambridge, UK), a lateral marker, and confocal x-z microscopy analysis. After washing cells with Hank's Balanced Salt Solution (HBSS) the Transwell inserts were placed in 24-well plates filled with 600 µl of HBSS, and 70 kDa FITC-dextran (Molecular Probes Inc.) diluted in HBSS (100 µl, 100 µg/ml) was added to the upper chamber. The ability of FITC-dextran to diffuse through the monolayers was evaluated after 1 hour of incubation by measuring a 100 µl aliquot from the lower chamber in a Fluostar Optima flourometer (BMG Labtechnologies GmbH, Offenburg, Germany). For analysis, the values obtained were interpolated into a standard curve. In calcium depletion experiments, control cells were washed with calcium and magnesium-supplemented PBS (MidiMed, Boussens, France) before the incubation with FITC-dextran diluted in the same buffer; in order to remove calcium, PBS supplemented with 1 mM EGTA was employed to wash cells and dilute FITC-dextran.

Statistical analysis

Results are given as mean ± SD. After performing normality tests, comparison between groups was done by using the nonparametric Mann-Whitney U-test or the Paired t-test as indicated. *P* less that 0.05 was considered statistically significant. The statistical program GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA) was used.

Supplementary Figures

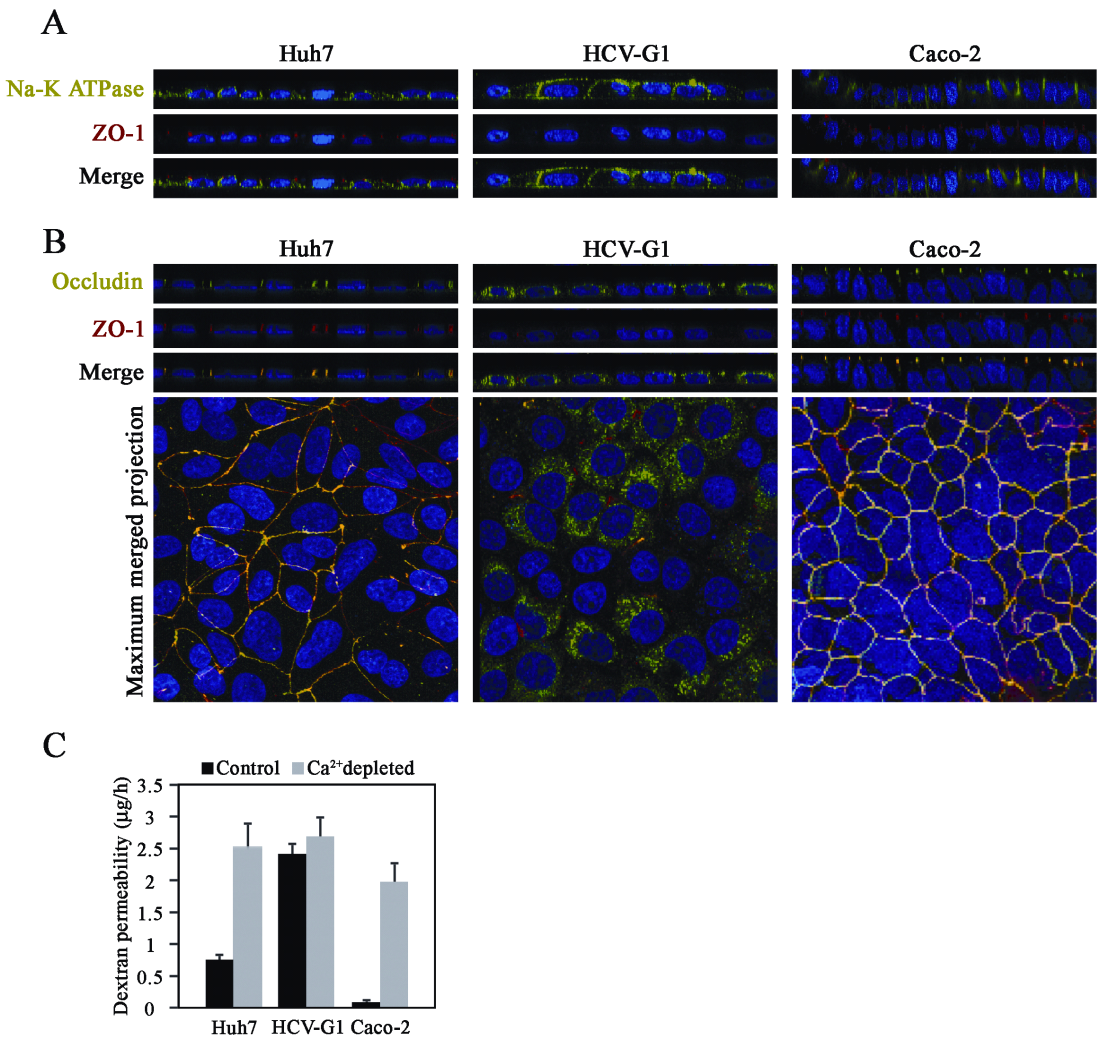


Figure S1. Analysis of polarity and TJ-associated proteins in Huh7, HCV-G1 and Caco-2 cells. (A) Huh7 (left), HCV-G1 (center) or Caco-2 (right) cells were grown on Transwell filters for 72h (Huh7 and HCV-G1 cells) or 10 days (Caco-2 cells) and processed for immunofluorescence with anti-Na-K ATPase (lateral marker [34], green) and ZO-1 (red) Abs, being shown the x-z sections. Z-sections were compiled by taking 0.6 µm steps through each x-y section. (B) Localization of occludin (green) and ZO-1 (red) in Transwell-cultured Huh7 (left), HCV-G1 (center) and Caco-2 (right) cells was analyzed by immunofluorescence (top, x-z sections; bottom, merged projection of confocal stacks). (C) Paracellular permeability of control or calcium-depleted Huh7, HCV-G1 and Caco-2 cells was determined as described in Supp. M&M and Fig. 1 legend. Results are expressed as the mean value ± SD of six experiments.

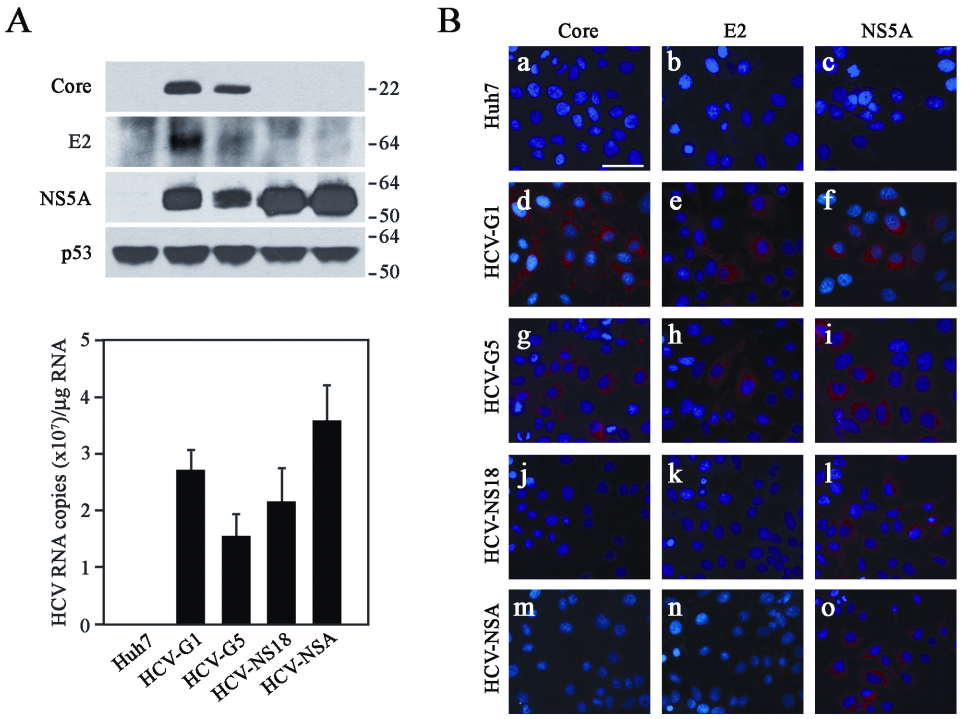


Figure S2. Characterization of genomic and subgenomic HCV replicon-containing clones. (A) Top: Western blot analysis of the HCV proteins core, E2 and NS5A expression in Huh7 cells and clones harbouring the genomic replicon I_{389}/Core-3'/5.1 (HCV-G1, HCV-G5) or the subgenomic replicon I_{377}/NS3-3' (HCV-NS18, HCV-NSA). After 48 hours of culture, cell lysates were analyzed for core, E2 and NS5A expression by Western blot. Analysis of p53 protein levels determined relative equal amounts of protein in all lanes. Molecular weight markers are indicated on the right (in kilodaltons). Bottom: HCV RNA levels. Samples of 1 µg RNA were analyzed by real-time RT-PCR using specific primers to determine HCV RNA levels. Histone H3 mRNA levels were used for sample normalization. Data are expressed as HCV RNA copies/µg total RNA. Data are represented as the mean value ± SD of four experiments. (B) Immunofluorescence analysis of core, E2 and NS5A expression in Huh7 (a-c), genomic [HCV-G1 (d-f), HCV-G5 (g-i)] and subgenomic clones [HCV-NS18 (j-l), HCV-NSA (m-o)]. Cells were fixed and processed for immunostaining with anti-core (a, d, g, j, m), anti-E2 (b, e, h, k, n) and anti-NS5A (c, f, i, l, o) specific Abs. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to localize nuclei. Bar, 50 µm. Results are representative of five separate experiments.

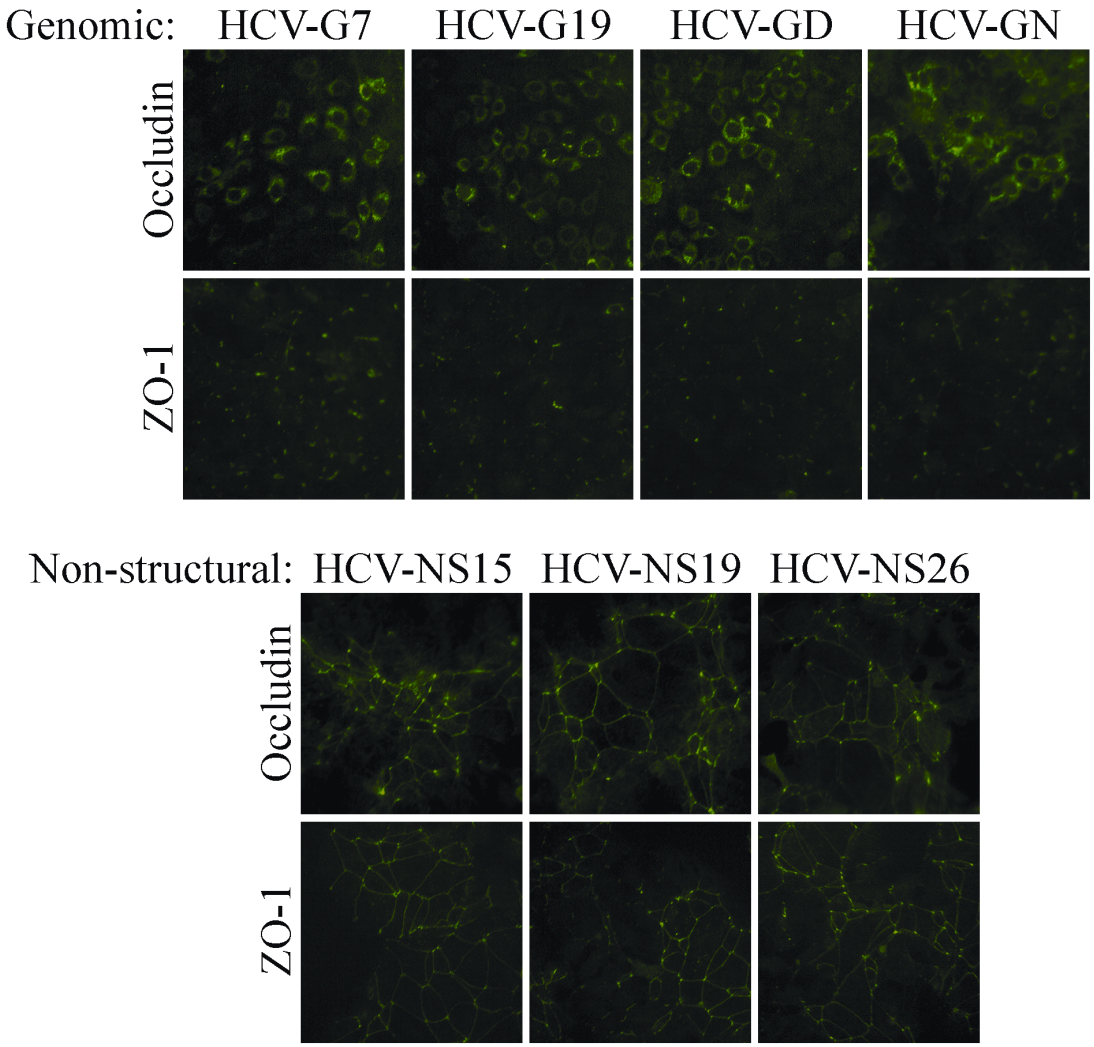


Figure S3. Immunofluorescence analysis of TJ-associated proteins in additional replicon clones. Occludin and ZO-1 localization in the rest of genomic (top) and subgenomic (non-structural, bottom) HCV isolated clones after 48 hours of cell culture. Results are representative of five separate experiments.

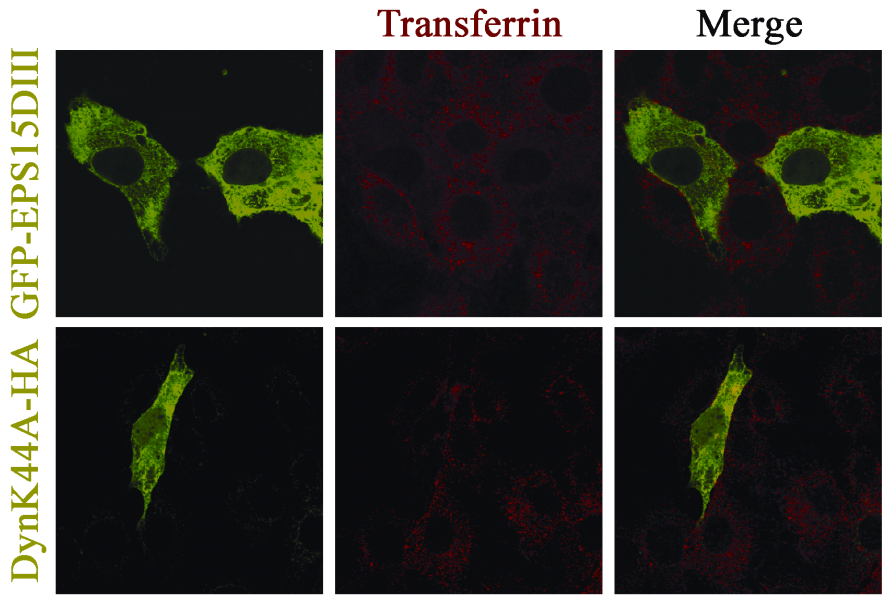


Figure S4. Effect of dominant negative constructs inhibiting clathrin and clathrin/caveolin endocytic pathways on HCV-G1 cells. Cells were transiently transfected with plasmids harbouring dominant negative constructs inhibiting clathrin (top, GFP-EPS15DIII) or clathrin/caveolin endocytic pathways (bottom, Dyn2K44A-HA). After 48 hours of culture, cells were incubated with Texas Red-labelled transferrin (see Supp. M&M) and either mounted (for GFP-EPS15DIII transfectants) or processed for immunofluorescence with anti-HA and Alexa 488-conjugated goat anti-mouse (for Dyn2K44A-HA transfectants). Transferrin internalization (red) was severely impaired in transfected cells (green).

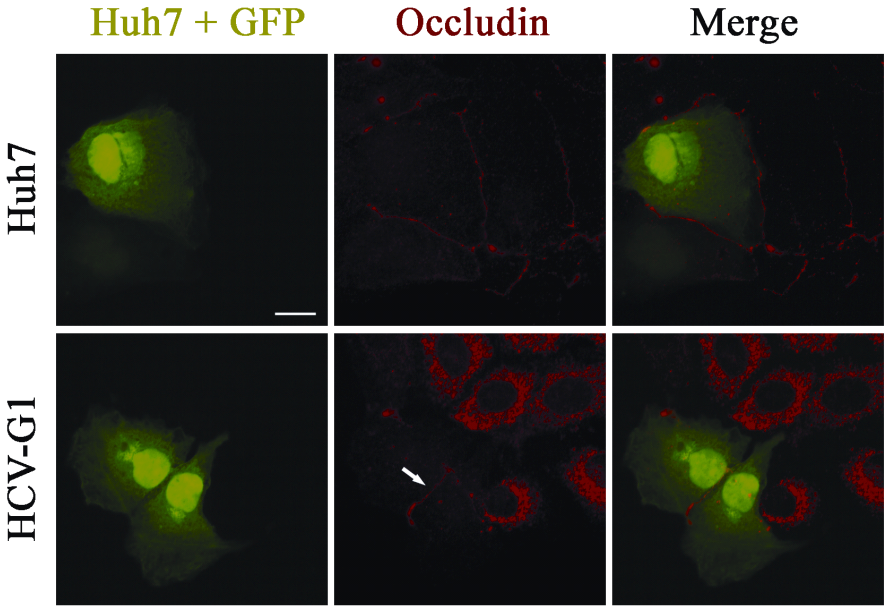


Figure S5. Analysis of occludin localization in Huh7 cells co-cultured with genomic replicon-containing clones. After transfection of Huh7 cells with pEGFP-C1, GFP-expressing cells were sorted, mixed with non-transfected Huh7 (top) or HCV-G1 (bottom) cells and seeded onto glass coverslips. After 48 hours of culture, localization of occludin was analyzed by immunofluorescence using anti-occludin (red) specific Ab. Bar, 20 µm. Arrow: the intercellular occludin staining between Huh7 cells (expressing GFP) is not altered when co-cultured with HCV-G1 cells.

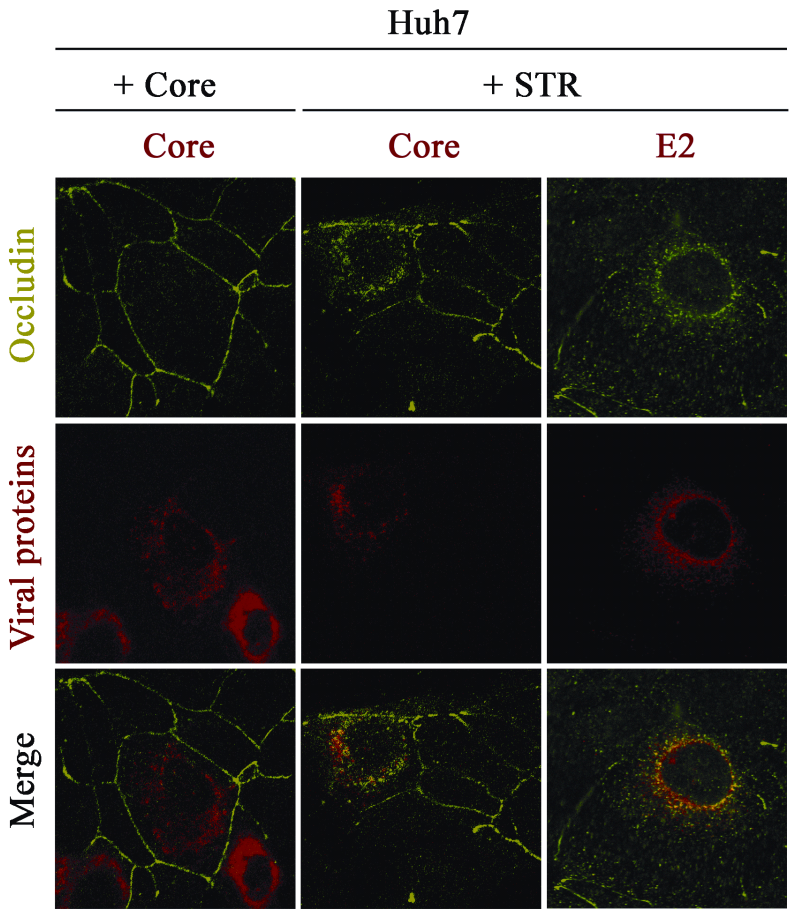


Figure S6. Effect of HCV structural proteins on the localization of Huh7 TJ-associated proteins. Parental Huh7 cells were transfected with plasmids coding for HCV structural proteins (+ STR) or core protein (+ Core) and the distribution of occludin and viral proteins was analyzed by confocal immunofluorescence. Transfected Huh7 cells were grown on coverslips for 48 hours, fixed and processed for double-label immunofluorescence using anti-occludin (green) and anti-core or anti-E2 (red) Abs. Representative confocal images of cells are shown.

Supplementary References

30. Hope RG, McLauchlan J. Sequence motifs required for lipid droplet association and protein stability are unique to the hepatitis C virus core protein. J Gen Virol. 2000;81(Pt 8):1913-1925.

31. Gomez-Gonzalo M, Benedicto I, Carretero M, Lara-Pezzi E, Maldonado-Rodriguez A, Moreno-Otero R, et al. Hepatitis C virus core protein regulates p300/CBP co-activationv function. Possible role in the regulation of NF-AT1 transcriptional activity. Virology 2004;328(1):120-130.

32. Krönke J, Kittler R, Buchholz F, Windisch MP, Pietschmann T, Bartenschlager R, Frese M. Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. J Virol 2004;78(7):3436-46.

33. Yang S, Delgado R, King SR, Woffendin C, Barker CS, Yang ZY et al. Generation of retroviral vector for clinical studies using transient transfection. Hum Gene Ther 1999;10:123–132.

34. Shoshani L, Contreras RG, Roldán ML, Moreno J, Lázaro A, Balda MS et al. The polarized expression of Na+,K+-ATPase in epithelia depends on the association between beta-subunits located in neighboring cells. Mol Biol Cell. 2005;16(3):1071-81.

ANEXO 2

ANEXO 2

La proteína asociada a TJs ocludina es necesaria para la infección por HCV participando en un paso posterior a la unión inicial del virus a la célula diana.
J Virol. 2009; 83:8012-20.

La observación previa de la existencia de una interacción intracelular entre la proteína E2 de HCV y ocludina nos llevó a pensar que quizás dicha asociación también pudiera tener lugar en el exterior de la célula. Esta situación sería viable ya que la topología natural de ocludina es la membrana plasmática y E2 se encuentra anclada en la envuelta del virus. Por ello, ambas proteínas podrían interaccionar durante alguna fase de la entrada de HCV en la célula, de tal manera que ocludina podría funcionar como co-receptor viral. Esta hipótesis se apoya en el hecho de que claudina-1, otra proteína asociada a TJs, es necesaria para la infección por HCV. Por otra parte, se ha descrito que CAR y JAM-A, proteínas que también forman parte de las TJs, actúan como receptores de otros virus, y que la infección por Coxsackievirus depende de la presencia de ocludina en la célula diana.

Mediante transducción retroviral se generaron líneas celulares donde la expresión de ocludina se interfirió establemente. Estas líneas fueron infectadas con HCVcc y se pudo observar que ocludina era necesaria para la infección por HCV. Además, se comprobó que el silenciamiento de ocludina en células que contienen replicones genómicos de HCV no afectaba a la replicación viral. Estos datos sugerían que la fase de la infección en la que ocludina estaba implicada era la entrada del virus en la célula. Esta hipótesis se pudo demostrar mediante el uso de HCVpp, observándose además que otras proteínas asociadas a TJs, como JAM-A o ZO-1, no participaban en el proceso infectivo. Estos datos indicaban que ocludina estaba implicada de forma específica en alguna fase de la entrada de HCV en la célula huésped.

Para indagar en el mecanismo a través del cual ocludina posibilitaba la entrada viral, primero se comprobó si su presencia era necesaria para la unión inicial del virus a la célula, comprobándose que no lo era. Seguidamente, se descartó la posibilidad de que la disminución de los niveles de ocludina pudiera alterar la expresión o la localización subcelular de otros co-receptores de HCV, lo que indicó una implicación directa de ocludina en la entrada de HCV. Finalmente, mediante ensayos de fusión célula-célula se pudo determinar un posible papel de ocludina en el proceso de fusión de

membranas mediada por las glicoproteínas de la envuelta de HCV. Todos estos datos indican que ocludina participa en la entrada de HCV en la célula diana, posiblemente durante una fase tardía de la misma.

JOURNAL OF VIROLOGY, Aug. 2009, p. 8012–8020 Vol. 83, No. 16
0022-538X/09/$08.00+0 doi:10.1128/JVI.00038-09

The Tight Junction-Associated Protein Occludin Is Required for a Postbinding Step in Hepatitis C Virus Entry and Infection▽

Ignacio Benedicto,[1,2] Francisca Molina-Jiménez,[1,2] Birke Bartosch,[3,4,10,11] François-Loïc Cosset,[3,10,11] Dimitri Lavillette,[3,10,11] Jesús Prieto,[2,5,6] Ricardo Moreno-Otero,[2,7] Agustín Valenzuela-Fernández,[8] Rafael Aldabe,[2,6] Manuel López-Cabrera,[1,2,9] and Pedro L. Majano[1,2]*

Molecular Biology Unit, Hospital Universitario de la Princesa, Madrid, Spain[1]; CIBER-ehd, Instituto de Salud Carlos III, Madrid, Spain[2]; Université de Lyon, UCB-Lyon1, IFR128, Lyon F-69007, France[3]; Hospices Civils de Lyon (HCL), Lyon, France[4]; Liver Unit, Clínica Universitaria, Universidad de Navarra, Pamplona, Spain[5]; Division of Gene Therapy and Hepatology, Centro de Investigación en Medicina Aplicada, CIMA, Pamplona, Spain[6]; Liver Unit, Hospital Universitario de la Princesa, Madrid, Spain[7]; Cellular and Viral Immunology Laboratory, Pharmacology Unit, Departamento de Medicina Física y Farmacología, Facultad de Medicina, Instituto de Tecnologías Biomédicas, Universidad de La Laguna, and Instituto Canario de Investigación del Cáncer (ICIC), Tenerife, Spain[8]; Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas (CSIC), Cantoblanco, Madrid, Spain[9]; INSERM, U758, Lyon F-69007, France[10]; and Ecole Normale Supérieure de Lyon, Lyon-F-69007, France[11]

Received 8 January 2009/Accepted 28 May 2009

The precise mechanisms regulating hepatitis C virus (HCV) entry into hepatic cells remain unknown. However, several cell surface proteins have been identified as entry factors for this virus. Of these molecules, claudin-1, a tight junction (TJ) component, is considered a coreceptor required for HCV entry. Recently, we have demonstrated that HCV envelope glycoproteins (HCVgp) promote structural and functional TJ alterations. Additionally, we have shown that the intracellular interaction between viral E2 glycoprotein and occludin, another TJ-associated protein, could be the cause of the mislocalization of TJ proteins. Herein we demonstrated, by using cell culture-derived HCV particles (HCVcc), that interference of occludin expression markedly reduced HCV infection. Furthermore, our results with HCV pseudotyped particles indicated that occludin, but not other TJ-associated proteins, such as junctional adhesion molecule A or zonula occludens protein 1, was required for HCV entry. Using HCVcc, we demonstrated that occludin did not play an essential role in the initial attachment of HCV to target cells. Surface protein labeling experiments showed that both expression levels and cell surface localization of HCV (co)receptors CD81, scavenger receptor class B type I, and claudin-1 were not affected upon occludin knockdown. In addition, immunofluorescence confocal analysis showed that occludin interference did not affect subcellular distribution of the HCV (co)receptors analyzed. However, HCVgp fusion-associated events were altered after occludin silencing. In summary, we propose that occludin plays an essential role in HCV infection and probably affects late entry events. This observation may provide new insights into HCV infection and related pathogenesis.

Hepatitis C virus (HCV) is a small enveloped positive-strand RNA virus that belongs to the *Flaviviridae* family (20). More than 80% of acute infections become chronic, which eventually progress to cirrhosis and hepatocellular carcinoma (28). HCV infects mainly hepatocytes, but the precise mechanisms of infection are largely unknown (11). The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer in which the two envelope glycoproteins (HCVgp), E1 and E2, are anchored as a heterodimer and play a major role in HCV entry (20). The development of an infectious cell culture model based on the production of infective HCV particles (cell culture-derived HCV particles [HCVcc]) (34) and the generation of HCV pseudotyped retroviral particles (HCVpp) (4) have provided powerful tools to study the HCV cycle. HCV entry is a complex multistep process that requires the presence of several factors. There are multiple pieces of evidence for the involvement of host cell proteins in HCV entry, including glycosaminoglycans, the low-density lipoprotein receptor, scavenger receptor class B type I (SR-BI), and the tetraspanin CD81 (11). Recently, claudin-1, a tight junction (TJ) component, has been identified as a coreceptor required for a late step in HCV entry (13).

TJs are major components of cell-cell adhesion complexes and are composed of integral membrane proteins, including occludin and claudins, which associate with actin cytoskeleton-interacting proteins, such as zonula occludens protein 1 (ZO-1) (2). These structures maintain cell polarity, separating apical from basolateral membrane domains, and form a paracellular barrier that allows the selective passage of certain solutes (2). In hepatocytes, TJs seal the bile canaliculi and form the intercellular barrier between bile and blood (12). Recently, we have shown that TJ-associated proteins occludin and claudin-1 disappeared from their normal localization in both HCV-infected and genomic HCV replicon-containing Huh7 cells. Furthermore, TJ function was also altered in these cells (5). In this matter, we have reported an intracellular interaction be-

* Corresponding author. Mailing address: Unidad Biología Molecular, Hospital Universitario de la Princesa, C/ Diego de León 62, 28006 Madrid, Spain. Phone: 34-91-5203462. Fax: 34-91-3093911. E-mail: pmajano.hlpr@salud.madrid.org.

▽ Published ahead of print on 10 June 2009.

tween E2 and occludin (5). Moreover, it has been reported that claudin-1 and several TJ-associated proteins, such as coxsackievirus and adenovirus receptor (35) and junctional adhesion molecule (JAM) (3), act as virus (co)receptors. Since coxsackievirus entry across epithelial TJs requires occludin (10), we have explored the role of occludin in HCV infection.

MATERIALS AND METHODS

Cell culture, generation of HCV replicon-containing clones, and HCVcc infection. Huh7, PLC/PRF/5, and 293T cells and their derivatives were grown at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin. Huh7 cells expressing full-length or subgenomic genotype 1b (Con1; EMBL database accession number AJ238799) HCV replicons were established as previously described (5). For HCVcc infection assays, cells were grown on 96-well plates (3×10^4 cells/cm^2) for 24 h, spin infected with HCV isolate JFH1 (multiplicity of infection of 0.1) (34) at 1,200 × *g* for 2 h (36) and processed 4 days postinfection for real-time reverse transcription-PCR (RT-PCR) or immunofluorescence (see below).

Transgene and short hairpin RNA (shRNA) retroviral transfer. Full-length human claudin-1 cDNA (GenBank accession number NM_021101) was cloned into the SwaI site of the pRV-IRES-Neo retroviral expression vector (Genetrix S. L., Madrid, Spain). This construct was employed to generate the 293T-claudin-1 cell line.

cDNA oligonucleotides containing the sequence corresponding to nucleotides 1412 to 1430 of human occludin cDNA (GenBank accession NM_002538) were annealed and inserted between the BglII and HindIII sites of pSUPER.retro-.puro and pSUPER.retro.neo+GFP (GFP stands for green fluorescent protein) (Oligoengine, Seattle, WA) according to the manufacturer's instructions. This sequence is identical to nucleotides 1480 to 1498 of canine occludin, which has been reported to be an efficient target for occludin knockdown in MDCK cells (37). Generation of the control shRNA retroviral vector, production of retroviral particles, infection, and puromycin selection of target cells were performed as previously described (5). Occludin silencing in HCV replicon-containing clones was performed by transient transfection with the pSUPER.retro.neo+GFP versions of control and occludin shRNA due to the fact that HCV replication was reduced two- to fourfold after retroviral infection and puromycin selection (our unpublished observations). For this purpose, transfections were performed with 2 μg of the indicated plasmids employing Lipofectin (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 24 h of culture, GFP-expressing cells were purified by flow cytometry with a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ), and 24 h later, RNA was extracted for real-time RT-PCR analysis (see below).

siRNAs and transfections. Cells were transfected using the Dharmafect 4 protocol with a mix of two On-Targetplus small interfering RNAs (siRNAs) (100 nM each) directed against human occludin (5′-GAAGAAAGAUGGACAGGUA-3′ and 5′-GUACUGGGGUUCAUGAUUA-3′), JAM-A (5′-GGAUAGUGAUGCCUACGAA-3′ and 5′-CGAGUAAGAAGGUGAUUUA-3′), ZO-1 (5′-GAGAAGAAGUGACCAUAUU-3′ and 5′-CUACACUGAUCAAGAACUA-3′), or a nonspecific control siRNA (Dharmacon, Lafayette, CO). After 48 h, cells were reseeded (3×10^4 cells/cm^2) and 24 h later infected with luciferase-based pseudoparticles or lysed to check knockdown efficiency by Western blotting (see below).

HCVcc binding assays. Cells were grown on 24-well plates (3×10^4 cells/cm^2) for 24 h. After the cells were washed with cold culture medium, they were incubated with HCVcc (multiplicity of infection of 0.1) supplemented with 20 mM HEPES for 1 h at 4°C with gentle rocking. Where indicated, heparin (250 μg/ml) was added. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and processed for RT-PCR (see below).

RT-PCR. RNA extraction, RT, and quantitative PCR were performed as previously described (5). Occludin-specific primers used were 5′-TGCATGTTCGACCAATGC-3′ and 5′-AAGCCACTTCCTCCATAAGG-3′ (1).

Western blots. Cells were grown on six-well plates (3×10^4 cells/cm^2) for 24 h, lysed on the plate with 100 μl of Laemmli buffer, and boiled for 5 min. Western blotting was carried out as previously described (5) with the following antibodies: polyclonal antibodies antioccludin, anti-claudin-1, anti-ZO-1, anti-JAM-A (Zymed, San Francisco, CA) and anti-SR-BI (Novus Biologicals, Inc., Littleton, CO) and monoclonal antibodies anti-CD81 (clone 5A6) and anti-p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Plasmid constructs. The MLV-GFP, CMV-Gag-Pol, and phCMV-RD (encoding the feline endogenous virus RD114 glycoproteins) vectors and the plasmids coding for HCV E1E2 glycoproteins (genotypes 1a and 1b) have been previously described (4). The pVSV-G construct (encoding the vesicular stomatitis virus [VSV] G protein) was purchased from Clontech Laboratories Inc., Mountain View, CA. The pNL4-3.Luc.R-E- plasmid was obtained through the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) from Nathaniel Landau (8, 15). The pXP1LTRwt construct (human immunodeficiency virus type 1 [HIV-1] long terminal repeat [LTR]-luciferase reporter plasmid) has been previously described (14). The pRL-null reporter plasmid was purchased from Promega, Madison, WI.

Production of pseudoparticles and infection assays. GFP-coding pseudoparticles were generated as previously described (4). Production of luciferase-based pseudoparticles was performed by transfection of 293T cells with equal amounts (10 μg) of pNL4-3.Luc.R-E- and either HCV E1E2, phCMV-RD, pVSV-G, or empty vector by the calcium phosphate method. The medium was replaced 16 h after transfection. Supernatants containing the pseudoparticles were harvested 24 h later, filtered through 0.45-μm-pore-size membranes, and used in infection assays. Target cells were grown on 96-well plates (3×10^4 cells/cm^2) for 24 h and infected as previously described (4). 293T-derived cells were seeded on plates coated with poly-L-lysine (Sigma, St. Louis, MO). For GFP-based pseudoparticles, infection was enhanced by spinoculation (centrifugal inoculation) (see above), and the percentage of GFP-positive cells was determined using a FACSCalibur flow cytometer (BD Biosciences). Luciferase activity was measured with the luciferase assay system (Promega) according to the manufacturer's instructions and determined in a Sirius single tube luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany).

Immunofluorescence analysis and confocal microscopy. Cells were grown on coverslips (3×10^4 cells/cm^2) for 24 h and processed for immunofluorescence as previously described (5). 293T-derived cells were seeded on coverslips coated with poly-L-lysine. The antibodies used were the same as for Western blots (see above), monoclonal anti-HCV core (clone C7-50; Affinity BioReagents, Goleen, CO) and polyclonal anti-ZO-1 (Zymed). A monoclonal antioccludin antibody (Zymed) was used for double-label immunofluorescence when needed.

Cell surface protein biotinylation. A previously described protocol for cell surface protein biotinylation (26) was followed with minor modifications. After 24 h of culture (3×10^4 cells/cm^2), cells were washed three times with ice-cold calcium- and magnesium-supplemented phosphate-buffered saline (PBS-CM) (MidiMed, Boussens, France) and incubated with 0.5 mg/ml Sulfo-NHS-SS-Biotin [sulfosuccinimidyl 2-(biotinamido)-ethyl-1, 3-dithiopropionate] (Pierce, Rockford, IL) in PBS-CM for 45 min at 4°C. Cells were washed three times with ice-cold Tris-buffered saline and lysed in buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% NP-40, and EDTA-free Halt protease inhibitor cocktail (Pierce) for 30 min on ice. After sonication for 1 min in a Soniprep 150 (MSE Ltd., Crawley, United Kingdom), lysates were cleared by centrifugation (23,440 × *g*) for 15 min at 4°C, and the protein concentration was measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Cleared lysates (1 mg) were incubated with 100 μl of 50% immobilized NeutrAvidin protein (Pierce) overnight at 4°C with mixing. Samples were centrifuged (1 min, 500 × *g*), and precipitates were washed three times with ice-cold lysis buffer, resuspended in Laemmli buffer, and boiled for 10 min. Bound proteins were separated on a sodium dodecyl sulfate-polyacrylamide gel and analyzed by Western blotting using the indicated antibodies.

Cell-cell fusion assays. Cell-cell fusion experiments were performed essentially as previously described (19). 293T cells were transduced with occludin shRNA-coding retroviral particles and selected with puromycin (see above). 293T-occludin shRNA "donor" cells (5×10^5 cells/well seeded into the wells of six-well plates [wells coated with poly-L-lysine 24 h prior to transfection]) were cotransfected using Lipofectamine (Invitrogen) with 100 ng of pXP1LTRwt plasmid and either 2 μg of empty vector, 2 μg of phCMV-H77, or 0.4 μg of pVSV-G plus 1.6 μg of empty vector. After 24 h, transfected cells were reseeded (2×10^4 cells/well) in 24-well plates. Huh7-Tat indicator cells (19) were transfected with control or occludin siRNAs (see above); after 48 h, cells were detached and added (8×10^4 cells/well) to the transfected 293T-occludin shRNA "donor" cells. After 24 h of cocultivation, cells were washed with PBS, incubated for 5 min in citric acid buffer (15 mM citric acid, 150 mM NaCl) at pH 5, and washed three times with medium. The luciferase activity was measured 24 h later as described above. In order to test whether occludin knockdown affected Tat-associated transcriptional activity, control and occludin siRNA-transfected Huh7-Tat cells were seeded in the wells of 24-well plates (6×10^4 cells/well) 24 h after siRNA transfection, cultured overnight, and transiently cotransfected with 20 ng of pXP1LTRwt, 4 ng of pRL-null, and 200 ng of carrier plasmid. Parental Huh7 cells (not expressing Tat) were transfected in parallel and used as a control for Tat-dependent HIV-1 LTR induction. After 48 h, luciferase activity was

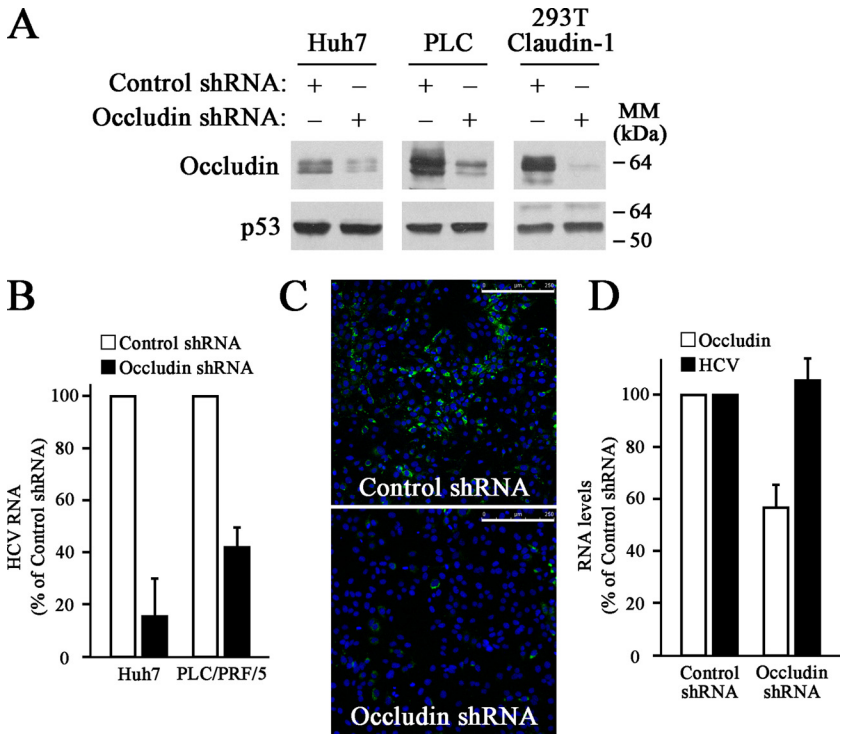


FIG. 1. HCVcc infection is impaired by occludin knockdown. (A) Occludin was silenced using shRNA technology on Huh7, PLC/PRF/5 (PLC), and 293T-Claudin-1 cells. Occludin knockdown was confirmed by Western blotting using antioccludin and anti-p53 (loading control) antibodies. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the blots. Results are representative of two independent experiments. (B) Cells were infected with HCVcc, and 1-μg RNA samples were analyzed by real-time RT-PCR using specific primers to determine HCV RNA levels. Histone H3 mRNA levels were used for sample normalization. Data are expressed as HCV RNA levels relative to control shRNA-transduced cells. Data are represented as the mean values plus standard deviations (SD) (error bars) from three experiments. (C) HCVcc-infected Huh7 cells were processed for immunostaining using an antibody directed specifically against HCV core protein (green). The merged image with the nuclei stained with 4′,6′-diamidino-2-phenylindole (DAPI) (blue) is shown. Results are representative of two separate experiments. Bars, 250 μm. (D) Occludin was silenced using shRNA technology on Huh7 clones harboring the genomic replicon I389/Core-3′/5.1 (HCV-G1) or the subgenomic replicon I377/NS3-3′ (HCV-NSA). Occludin and HCV RNA levels were determined by real-time RT-PCR as described in Materials and Methods. Data are expressed as the mean values plus SD (error bars) of the results obtained with both clones.

measured with the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

RESULTS

The TJ-associated protein occludin is relevant to HCV infection. Recently, it has been suggested that claudin-1 is implicated in HCV infection (13). To evaluate the contribution of the TJ-associated protein occludin to HCV infection, we used a retroviral knockdown approach with control or occludin-specific shRNAs in both Huh7 and PLC/PRF/5 human hepatocyte-derived cell lines. Furthermore, we included 293T-claudin-1 cells, derived from a nonhepatic cell line in which ectopically expressed claudin-1 renders 293T cells susceptible to HCVpp infection (13). Western blot analysis showed that occludin shRNA caused a marked downregulation of occludin expression in all cell lines tested (Fig. 1A). Then, we performed infection assays using HCVcc and evaluated productive infection by real-time RT-PCR. Occludin knockdown sharply reduced the susceptibility of Huh7 and PLC/PRF/5 cells to HCVcc infection compared to cells expressing control shRNA (Fig. 1B). The effect of occludin downregulation on HCVcc infection of 293T-claudin-1 cells could not be evaluated because no detectable levels of viral RNA were found after JFH-1 HCVcc infection (data not shown). This observation is consistent with a previous report and probably reflects the low permissiveness for initiation of HCV replication in 293T cells (32). These data were further confirmed by immunofluorescence analysis of viral protein core accumulation in control or occludin shRNA Huh7 cells (Fig. 1C).

In order to test whether occludin downregulation affects viral replication, we analyzed HCV RNA levels in Huh7 cells harboring genomic (HCV-G1) or subgenomic (HCV-NSA) replicons (5) after occludin knockdown. No differences in HCV RNA levels were found between control and occludin shRNA-expressing replicon-containing cells (Fig. 1D), indicating that HCV replication was not affected by occludin silencing. All together, these findings suggest that occludin expression is essential for HCV infection without affecting HCV replication.

Occludin knockdown impairs HCV entry. HCVpp carrying envelope proteins from different HCV genotypes have been validated for the study of HCV entry (4). HCVpp consist of full-length HCV envelope glycoproteins assembled onto retroviral core particles containing a retrovirus-derived genome harboring different reporter genes such as those encoding GFP or luciferase (4). To evaluate the contribution of occludin to

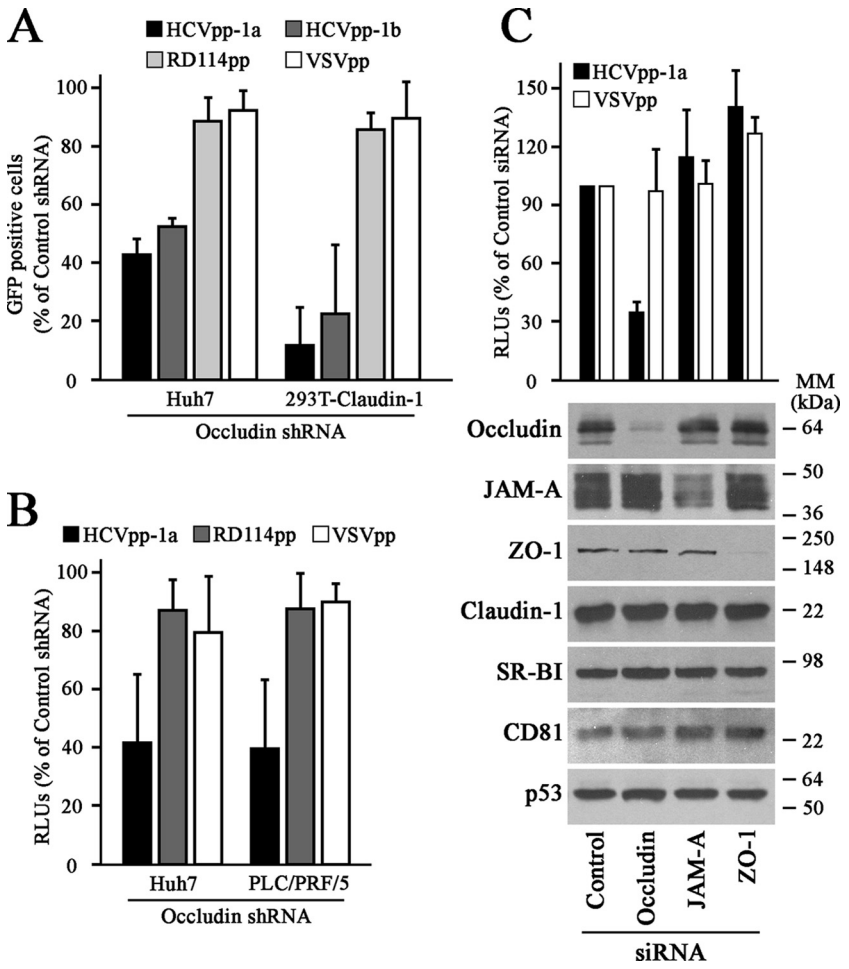


FIG. 2. Occludin knockdown alters HCV entry. (A) Infectivity of murine leukemia virus-derived GFP-coding pseudoparticles. Control or occludin shRNA-transduced cells were infected with HCVpp (genotypes 1a and 1b), VSVpp, or RD114pp. Data are expressed as percentages of GFP-positive cells relative to control shRNA-transduced cells. The percentage of GFP-positive cells observed with pseudoparticles bearing no envelope proteins (background reading) was subtracted from the values obtained with HCVpp, RD114pp, and VSVpp. Data are represented as the mean values plus standard deviations (SD) (error bars) of the results of three experiments performed in duplicate. (B) Infectivity of HIV-derived luciferase-coding pseudoparticles. Data are expressed relative to control shRNA-transduced cells. Background readings were always less than 2% of the values obtained with pseudoparticles containing envelope proteins. Data are represented as the mean values plus SD of the results of at least four experiments performed in triplicate. RLUs, relative luciferase units. (C) Effects of occludin-, JAM-A-, and ZO-1-specific siRNAs on pseudoparticle infectivity. PLC/PRF/5 cells were transiently transfected with control or specific siRNAs against occludin, ZO-1, and JAM-A. After 48 h, cells were reseeded (3×10^4 cells/cm^2), and 24 h later, the cells were infected with luciferase-based pseudoparticles or lysed to check knockdown efficiency by Western blotting using antioccludin, anti-JAM-A, anti-ZO-1, and anti-p53 (loading control) antibodies. Expression levels of claudin-1, SR-BI and CD81 were also monitored. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the blots. Data are expressed relative to control siRNA-transfected cells and represented as the mean values plus SD of the results of two independent experiments performed in triplicate.

HCV entry, control or occludin shRNA-transduced cells were challenged with GFP-coding HCVpp. Data showed that occludin knockdown resulted in a reduction of infectivity of both genotype 1a- and 1b-derived HCVpp without affecting VSV pseudotyped particles (VSVpp) or RD114 pseudotyped particles (RD114pp) infection levels (Fig. 2A). These data were further confirmed using luciferase-coding HCVpp (Fig. 2B).

The TJ multiprotein complex is composed by integral membrane proteins, such as JAM-A, which serves as a receptor for mammalian reovirus, and occludin, which associates with actin cytoskeleton-interacting proteins like ZO-1 (2). To evaluate the contributions of different TJ-associated proteins to HCV infection, Huh7 and PLC/PRF/5 cell lines were transiently transfected with occludin-, ZO-1-, and JAM-A-specific siRNAs. As expected, occludin knockdown after siRNA transfection resulted in a reduction of infectivity of HCVpp without affecting VSVpp infection levels on PLC/PRF/5 cells (Fig. 2C) or Huh7 cells (data not shown). In contrast, reduction of ZO-1 or JAM-A expression did not affect HCVpp infectivity (Fig. 2C). All together these findings suggest that occludin, but not other TJ-associated proteins, such as JAM-A or ZO-1, is essential for HCV entry.

Occludin knockdown does not affect initial HCV attachment or expression levels and subcellular localization of its (co)receptors. The HCV cell entry process starts with the binding of the viral particles to the surface of target cells via interactions

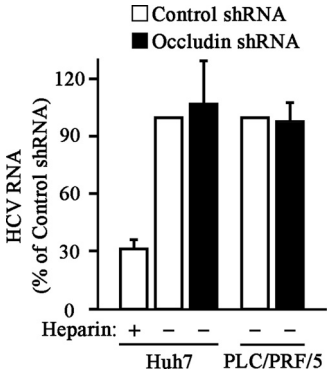


FIG. 3. Occludin knockdown does not affect HCV initial attachment to target cells. Control or occludin shRNA-transduced Huh7 or PLC/PRF/5 cells were washed with cold culture medium and incubated with HCVcc for 1 h at 4°C in the presence (+) or absence (−) of heparin (250 μg/ml). Unbound viral particles were removed, and total RNA was extracted. Samples (1 μg) of RNA were analyzed by real-time RT-PCR using specific primers to determine HCV RNA levels. Histone H3 mRNA levels were used for sample normalization. Data are expressed as HCV RNA levels relative to control shRNA-transduced cells in the absence of heparin. Data are represented as the mean values plus standard deviations (error bars) of the results of two independent experiments performed at least in duplicate.

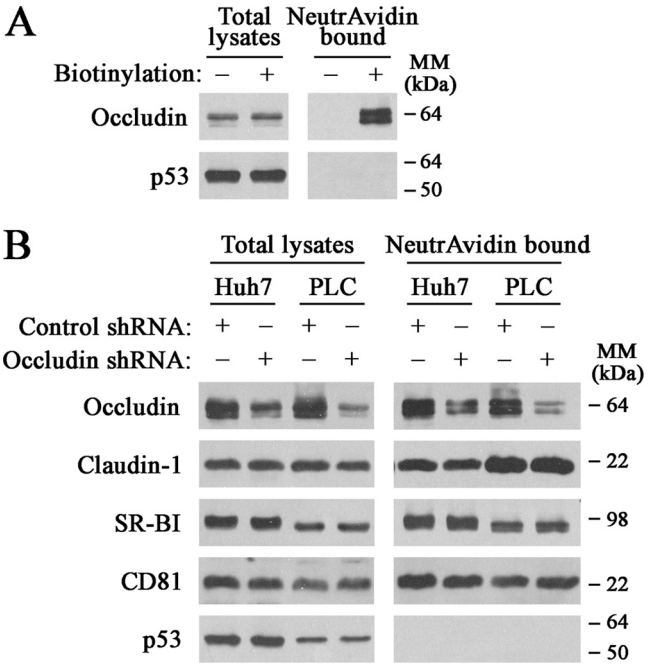


FIG. 4. Expression levels and cell surface localization of HCV (co)receptors are not altered after occludin knockdown. (A) After Huh7 cells were subjected to biotinylation (+), total lysates and NeutrAvidin-bound proteins obtained from mock-treated or biotin-treated cells were analyzed by Western blotting using antioccludin and anti-p53 antibodies. (B) Control or occludin shRNA-transduced cells were cell surface biotinylated. Western blot analysis of both total lysates and NeutrAvidin-bound proteins was performed using antioccludin, anti-claudin-1, anti-SR-BI, anti-CD81, and anti-p53 antibodies. Data shown are representative of at least two independent experiments. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the blots.

between HCVgp and specific cell surface receptors (11). We carried out HCVcc binding assays in order to examine whether occludin plays a role in initial cell binding events. Total RNA was extracted after incubating HCVcc with cells for 1 hour at 4°C. As shown in Fig. 3, we found no differences in HCVcc attachment between control and occludin shRNA-transduced cells (Huh7 and PLC/PRF/5 cells). Similar results were obtained by transient transfection of Huh7 cells with control or occludin siRNAs (data not shown). The specificity of HCVcc binding to cells was demonstrated by competition with heparin, a homolog of highly sulfated heparan sulfate, as a control to reduce HCV attachment to the cell surface (Fig. 3). These data suggest that occludin is not involved in the initial attachment of virions to target cells.

It has been suggested that the precise localization of HCV (co)receptors and their coordinate interactions with different proteins are required for productive HCV infection (11). In this context, we investigated whether occludin silencing affected expression levels and/or cell surface localization of HCV (co)receptors. When Huh7 cells were subjected to biotinylation of cell surface proteins, occludin was efficiently biotinylated and isolated by NeutrAvidin beads, and no occludin was detected when the biotinylation step was omitted (Fig. 4A). As expected, p53 was not detected in the NeutrAvidin precipitates, indicating no intracellular biotinylation (Fig. 4A). Western blot analysis of both total lysates and cell surface biotinylated extracts showed similar levels of HCV (co)receptors SR-BI, CD81, and claudin-1 between control and occludin shRNA-transduced cell lines (Fig. 4B). It is noteworthy that transient transfection of cells with occludin siRNA did not alter expression levels of HCV (co)receptors either (Fig. 2C).

Next we examined by confocal microscopy-based immunofluorescence analysis whether occludin silencing could alter the localization of the different HCV (co)receptors on cell surface, such as CD81 and SR-BI, and TJ-associated proteins claudin-1, occludin, and ZO-1 (Fig. 5). Given that the level of occludin silencing was not the same among all cells, we focused on fields where occludin expression was undetectable. We observed that in both control and occludin shRNA-transduced PLC/PRF/5 cells, claudin-1 and ZO-1 localized at the apical poles of lateral cell membranes, the typical distribution of these proteins in TJ-forming cells. Furthermore, CD81 and SR-BI appeared diffusely distributed across the plasma membrane independently of occludin silencing (Fig. 5). Similar results were obtained with Huh7 and 293T-claudin-1 cells (data not shown). In summary, these data indicate that occludin knockdown did not affect expression levels, cell surface localization, and spatial distribution of the HCV (co)receptors analyzed.

Occludin is implicated in HCV late entry events. Binding of HCV to target cells is followed by internalization of the viral particle by clathrin-mediated endocytosis (6). It has been established that VSV entry also relies on clathrin-mediated endocytosis (31). Since we observed that occludin shRNA did not induce any changes on VSVpp entry (Fig. 2), it is conceivable that occludin silencing did not affect clathrin-mediated endocytosis. However, in order to further confirm this issue, we examined whether transferrin uptake, which is a clathrin-dependent process (16), was altered after occludin knockdown. Experiments showed that transferrin internalization was not

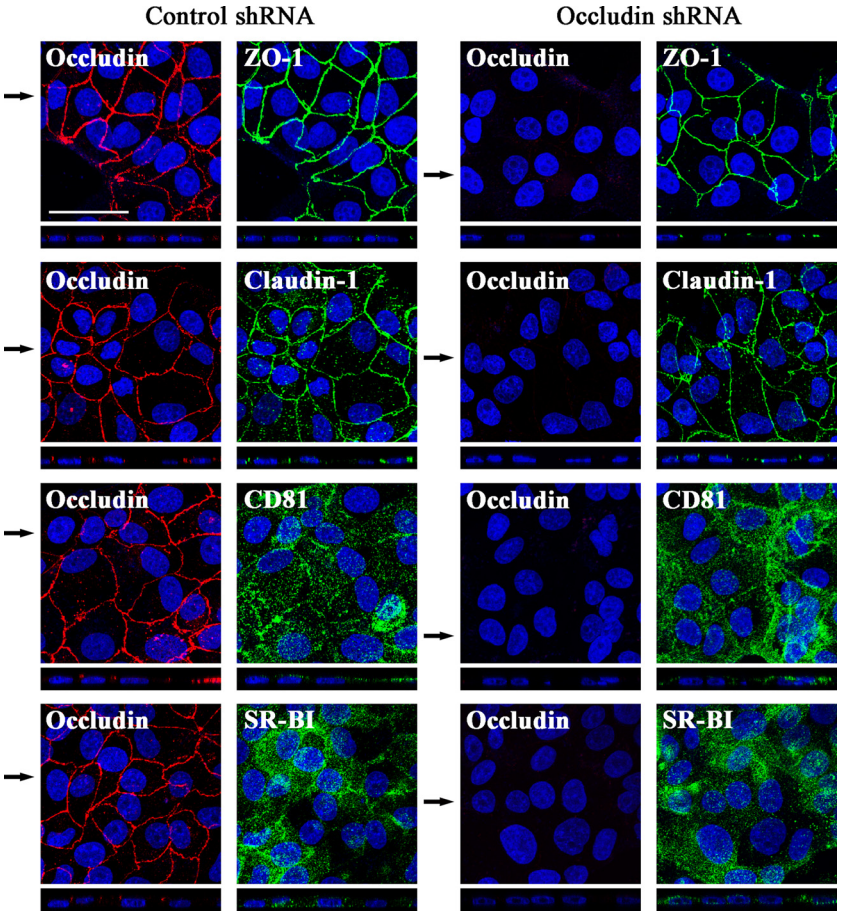


FIG. 5. Subcellular localization of HCV (co)receptors and TJ-associated protein ZO-1 is not affected by occludin silencing. Control or occludin shRNA-transduced PLC/PRF/5 cells were processed for immunofluorescence with antibodies against occludin, ZO-1, claudin-1, CD81, and SR-BI. Localization of occludin (red) and HCV (co)receptors or ZO-1 (green) was analyzed by immunofluorescence confocal analysis (the merged projection of confocal stacks is shown by the large labeled panel at the top, and the *x-z* section is shown by the thin panel at the bottom). Nuclei were stained with DAPI (blue). *z* sections were compiled by taking 0.5-μm steps through each *x-y* section. In order to clearly show the obtained results, due to the fact that occludin knockdown was not homogeneous among the occludin shRNA-transduced polyclonal cell population, cells with undetectable levels of occludin are shown. Images are representative of two independent experiments. The black arrows indicate the planes corresponding to the *x-z* sections shown. Bar, 50 μm.

affected in occludin shRNA-transduced Huh7 and 293T-claudin-1 cells (data not shown).

After HCV internalization, fusion has been proposed to occur within early endosomes (25), where the acidic pH triggers the fusion process, probably by inducing conformational changes in the envelope proteins (6, 17, 18, 33). To determine whether occludin is required for HCVgp-mediated membrane fusion, we carried out cell-cell fusion assays. In these experiments, either untransfected Huh7 cells or occludin and control siRNA-transfected Huh7-Tat "indicator" cells (19) were cocultured with 293T-occludin shRNA "donor" cells cotransfected with a HIV-1 LTR-luciferase reporter construct and plasmids coding for either HCV or VSV envelope proteins. Fusion between "donor" and "indicator" cells results in a Tat-mediated transactivation of the HIV-1 LTR measured as luciferase expression. Occludin knockdown in the "donor" cells was performed to avoid *cis*-interactions with HCVgp and *trans*-interactions with occludin expressed in the "indicator" cells. For similar reasons, we decided not to express claudin-1 in 293T "donor" cells. As expected, coculture of HCVgp-expressing "donor" cells with control siRNA-transfected Huh7-Tat cells produced almost 100-fold-more luciferase activity than parental Huh7 cells did (Fig. 6A), demonstrating effective cell-cell fusion and Tat-mediated transactivation of the HIV-1 LTR, thus validating the experimental setup. Interestingly, when HCVgp-expressing "donor" cells were used, occludin knockdown of Huh7-Tat cells resulted in a decrease of luciferase activity, indicating an impairment of HCVgp-dependent cell fusion (Fig. 6A). The specificity of occludin effects on HCVgp-mediated fusion was demonstrated by the fact that occludin knockdown of Huh7-Tat cells did not alter the fusion with "donor" cells expressing the VSV envelope protein (Fig. 6A). Occludin knockdown in Huh7-Tat cells was confirmed by Western blotting 48, 72, and 96 h after siRNA transfection, time points corresponding to the beginning of the coculture, the acid wash, and the measurement of luciferase activity, respectively (data not shown and Fig. 6B). Additionally, in order to rule out the possibility that occludin silencing affected Tat-associated transcriptional activity, control and occludin siRNA-transfected Huh7-Tat cells were transiently transfected

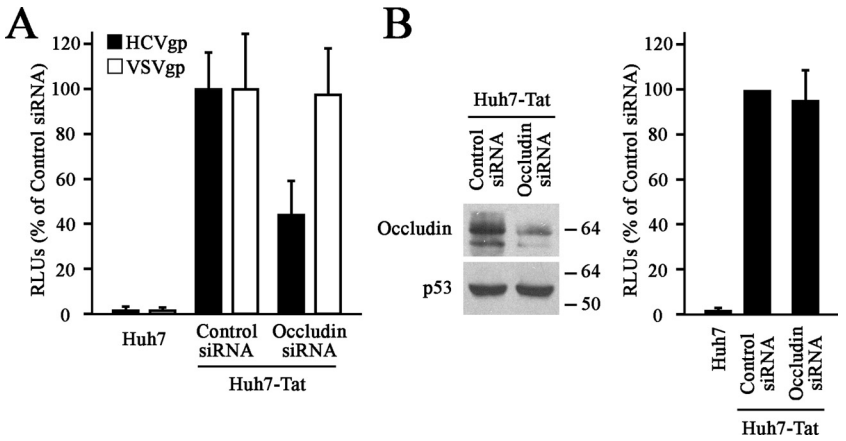


FIG. 6. Occludin knockdown affects HCVgp-mediated cell fusion. (A) 293T occludin-shRNA "donor" cells cotransfected with either empty vector, HCVgp, or VSVgp and a HIV-1 LTR-luciferase reporter construct were cocultured with control or occludin siRNA-transfected Huh7-Tat "indicator" cells. Parental Huh7 cells (not expressing Tat) were used as a control for Tat-dependent HIV-1 LTR induction. After 24 h, cells were treated at pH 5 for 5 min, and the luciferase activity induced by the fusion between "donor" and "indicator" cells was measured 24 h later. Unspecific fusion, measured as the luciferase activity obtained when using "donor" cells expressing no envelope proteins, was less than 15% of the values obtained with HCVgp (data not shown). The level of occludin knockdown in 293T-occludin shRNA "donor" cells was similar to that shown in Fig. 1 for shRNA-transduced 293T-claudin-1 cells (data not shown). Data are expressed relative to control siRNA-transfected Huh7-Tat cells and represented as the mean values plus standard deviations (SD) (error bars) of an experiment performed in triplicate. Results are representative of three independent experiments. RLUs, relative luciferase units. (B) Forty-eight hours after transfection of Huh7-Tat cells with control or occludin siRNA, cells were cotransfected with the HIV-1 LTR-luciferase reporter construct and the reporter plasmid pRL-null, which bears a promoterless *Renilla* luciferase gene and serves as control for transfection efficiency. Parental Huh7 cells were also included in the experiment. After 48 h of further culture, knockdown efficiency was checked by Western blotting and luciferase activity was measured. The positions of molecular mass markers (in kilodaltons) are shown to the right of the blots. *Renilla*-normalized data are expressed relative to control siRNA-transfected Huh7-Tat cells and represented as the mean values plus SD of an experiment performed in triplicate. Results are representative of three independent experiments.

with the HIV-1 LTR reporter plasmid, showing similar luciferase activities (Fig. 6B). All together, these results indicate that occludin is required for HCVgp-dependent cell fusion.

DISCUSSION

The most striking finding of this study is that the TJ-associated protein occludin is essential for HCV infection of several cell lines using both HCVcc and HCVpp. These data, along with the fact that occludin knockdown did not affect HCV replication, strongly suggest that occludin plays an essential role in HCV entry.

HCV entry is initiated by the binding of the particle to an attachment factor, which helps to concentrate viruses on the cell surface. After the initial attachment to the target cell, the virus binds to specific entry factors which are responsible for initiating a series of events that eventually lead to viral entry (11). Our data suggest that occludin is not involved in the initial attachment of virions to target cells, an infection step which probably depends on other virus (co)receptors, such as glycosaminoglycans and lipoprotein receptors.

Currently, it seems that HCV entry requires an unexpectedly large group of cellular (co)receptors, including SR-BI, CD81, and claudin-1 implicated at the postbinding stage (11). Our experiments suggest that the impairment of HCV entry after occludin knockdown was not due to a reduction of cell surface expression or spatial redistribution of the HCV (co)receptors analyzed. Furthermore, since occludin shRNA did not induce any changes in VSVpp infection and transferrin uptake, our data suggested that occludin knockdown did not affect clathrin-mediated endocytosis. Taken together, these findings suggest a rather direct and specific role of occludin in HCV entry.

HCV internalization is followed by membrane fusion, which likely occurs in early endosomes (25). HCVgp are resistant to inactivation by low pH (33), suggesting that HCVgp pH sensitivity occurs during cell entry probably mediated by cell surface molecules which could trigger conformational rearrangements and/or promote acid pH sensitivity of HCVgp. Occludin knockdown promoted a significant reduction of HCVgp-dependent cell-cell fusion, indicating its possible role in the fusion process or at an earlier step required to render the virus competent for low-pH-triggered entry. Interestingly, it has been shown that claudin-1 is required for HCVgp-dependent cell fusion (13), suggesting that the entry steps mediated by occludin and claudin-1 might involve similar mechanisms which could take place at the TJ. However, the relevance of TJs on HCV infection is still controversial and a matter of debate. After TJ disruption by calcium depletion, a significantly increased viral entry in Caco-2 cells (24) and an impaired HCV infection in Huh7 cells (7) have been observed. Our experiments showed that localization of TJ-associated proteins ZO-1 and claudin-1 was not significantly affected by occludin silencing. Furthermore, HCVpp infectivity was not altered by ZO-1 and JAM-A knockdown, suggesting that TJ function itself may not be required for HCV cell entry. However, further functional experiments will be required to determine whether TJs are necessary for HCV infection and whether occludin knockdown alters hepatocyte TJ function.

A number of studies suggest that viruses move laterally on the plasma membrane before being internalized (22). These

movements take place until the viruses have engaged sufficient receptors to initiate signaling events required for internalization. In the case of HCV, it has been suggested that the virus exploits CD81-mediated lateral migration to move to the TJs where the (co)receptor claudin-1 specifically localizes (7). A similar event has been demonstrated for the human coxsackievirus, whose binding on the apical surface of polarized cells triggers intracellular signals that permit virus to move to the TJ where it interacts with its primary receptor, coxsackievirus and adenovirus receptor, to enter target cells (9). Emerging evidence indicates that TJs are not an absolute and static barrier but rather a very dynamic cellular structure (29). In particular, occludin is recycled continuously even in epithelial cells with intact cell-cell contacts (30). Interestingly, the basolateral membrane is an obligatory intermediate in the transport of occludin to TJs (23). After HCV attachment, which presumably takes place on the basolateral membrane of hepatocytes in contact with the sinusoidal blood, occludin could be implicated in viral movement to the site where internalization occurs. Our previous recent results demonstrated an intracellular interaction between E2 and occludin (5). Thereby, it is plausible that the E2/occludin association could also take place extracellularly during a postbinding step probably to promote the subsequent viral internalization. Further studies are necessary to confirm this hypothesis.

It is important to point out that Liu and colleagues have also recently demonstrated that occludin participates in HCV infection (21). Furthermore, it has recently been shown that human occludin is an essential HCV cell entry factor, providing an important advance toward developing mouse models for HCV infection (27). Our data contribute important mechanistic details of the role of occludin in late steps of HCV entry. All together, these observations may provide new insights into HCV tropism, infection, and spreading that could help in understanding HCV-related pathogenesis.

ACKNOWLEDGMENTS

This work was supported in part by the following grants: (i) a grant from CIBER-ehd to R. Moreno-Otero, M. López-Cabrera, and P. Majano; (ii) grant SAF2007-61201 from the Ministerio de Educación y Ciencia to M. López-Cabrera; (iii) grant CP 03/0020 from Instituto Salud Carlos III; (iv) grant SAF2007-60667 from the Ministerio de Educación y Ciencia to P. Majano; and (v) a grant from the European Research Council (ERC-2008-AdG-233130 "HEPCENT") to F.-L. Cosset. I. Benedicto was financially supported by CIBER-ehd, and F. Molina-Jiménez was supported by ISCIII and FIB Hospital Universitario de la Princesa.

We express our gratitude to R. Bartenschlager, E. Muñoz, and T. Wakita for providing us with critical reagents. We also thank R. Samaniego for his technical assistance in confocal microscopy experiments.

REFERENCES

1. **Abe, T., E. Sugano, Y. Saigo, and M. Tamai.** 2003. Interleukin-1beta and barrier function of retinal pigment epithelial cells (ARPE-19): aberrant expression of junctional complex molecules. Investig. Ophthalmol. Vis. Sci. **44:**4097–4104.
2. **Aijaz, S., M. S. Balda, and K. Matter.** 2006. Tight junctions: molecular architecture and function. Int. Rev. Cytol. **248:**261–298.
3. **Barton, E. S., J. C. Forrest, J. L. Connolly, J. D. Chappell, Y. Liu, F. J. Schnell, A. Nusrat, C. A. Parkos, and T. S. Dermody.** 2001. Junction adhesion molecule is a receptor for reovirus. Cell **104:**441–451.
4. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
5. **Benedicto, I., F. Molina-Jimenez, O. Barreiro, A. Maldonado-Rodriguez, J. Prieto, R. Moreno-Otero, R. Aldabe, M. Lopez-Cabrera, and P. L. Majano.** 2008. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. Hepatology **48:**1044–1053.
6. **Blanchard, E., S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski, and Y. Rouille.** 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J. Virol. **80:**6964–6972.
7. **Brazzoli, M., A. Bianchi, S. Filippini, A. Weiner, Q. Zhu, M. Pizza, and S. Crotta.** 2008. CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. J. Virol. **82:**8316–8329.
8. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
9. **Coyne, C. B., and J. M. Bergelson.** 2006. Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. Cell **124:**119–131.
10. **Coyne, C. B., L. Shen, J. R. Turner, and J. M. Bergelson.** 2007. Coxsackievirus entry across epithelial tight junctions requires occludin and the small GTPases Rab34 and Rab5. Cell Host Microbe **2:**181–192.
11. **Dubuisson, J., F. Helle, and L. Cocquerel.** 2008. Early steps of the hepatitis C virus life cycle. Cell. Microbiol. **10:**821–827.
12. **Easter, D. W., J. B. Wade, and J. L. Boyer.** 1983. Structural integrity of hepatocyte tight junctions. J. Cell Biol. **96:**745–749.
13. **Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice.** 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature **446:**801–805.
14. **Gomez-Gonzalo, M., M. Carretero, J. Rullas, E. Lara-Pezzi, J. Aramburu, B. Berkhout, J. Alcami, and M. Lopez-Cabrera.** 2001. The hepatitis B virus X protein induces HIV-1 replication and transcription in synergy with T-cell activation signals: functional roles of NF-kappaB/NF-AT and SP1-binding sites in the HIV-1 long terminal repeat promoter. J. Biol. Chem. **276:**35435–35443.
15. **He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan, and N. R. Landau.** 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting $p34^{cdc2}$ activity. J. Virol. **69:**6705–6711.
16. **Hirst, J., and M. S. Robinson.** 1998. Clathrin and adaptors. Biochim. Biophys. Acta **1404:**173–193.
17. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100:**7271–7276.
18. **Koutsoudakis, G., A. Kaul, E. Steinmann, S. Kallis, V. Lohmann, T. Pietschmann, and R. Bartenschlager.** 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J. Virol. **80:**5308–5320.
19. **Lavillette, D., E. I. Pecheur, P. Donot, J. Fresquet, J. Molle, R. Corbau, M. Dreux, F. Penin, and F. L. Cosset.** 2007. Characterization of fusion determinants points to the involvement of three discrete regions of both E1 and E2 glycoproteins in the membrane fusion process of hepatitis C virus. J. Virol. **81:**8752–8765.
20. **Lindenbach, B. D., and C. M. Rice.** 2005. Unravelling hepatitis C virus replication from genome to function. Nature **436:**933–938.
21. **Liu, S., W. Yang, L. Shen, J. R. Turner, C. B. Coyne, and T. Wang.** 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. J. Virol. **83:**2011–2014.
22. **Marsh, M., and A. Helenius.** 2006. Virus entry: open sesame. Cell **124:**729–740.
23. **Matter, K., and M. S. Balda.** 1998. Biogenesis of tight junctions: the C-terminal domain of occludin mediates basolateral targeting. J. Cell Sci. **111:**511–519.
24. **Mee, C. J., J. Grove, H. J. Harris, K. Hu, P. Balfe, and J. A. McKeating.** 2008. Effect of cell polarization on hepatitis C virus entry. J. Virol. **82:**461–470.
25. **Meertens, L., C. Bertaux, and T. Dragic.** 2006. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. J. Virol. **80:**11571–11578.
26. **Morimoto, S., N. Nishimura, T. Terai, S. Manabe, Y. Yamamoto, W. Shinahara, H. Miyake, S. Tashiro, M. Shimada, and T. Sasaki.** 2005. Rab13 mediates the continuous endocytic recycling of occludin to the cell surface. J. Biol. Chem. **280:**2220–2228.
27. **Ploss, A., M. J. Evans, V. A. Gaysinskaya, M. Panis, H. You, Y. P. de Jong, and C. M. Rice.** 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature **457:**882–886.
28. **Poynard, T., M. F. Yuen, V. Ratziu, and C. L. Lai.** 2003. Viral hepatitis C. Lancet **362:**2095–2100.
29. **Shen, L., C. R. Weber, and J. R. Turner.** 2008. The tight junction protein complex undergoes rapid and continuous molecular remodeling at steady state. J. Cell Biol. **181:**683–695.
30. **Subramanian, V. S., J. S. Marchant, D. Ye, T. Y. Ma, and H. M. Said.** 2007.

Tight junction targeting and intracellular trafficking of occludin in polarized epithelial cells. Am. J. Physiol. Cell Physiol. **293:**C1717–C1726.

31. **Sun, X., V. K. Yau, B. J. Briggs, and G. R. Whittaker.** 2005. Role of clathrin-mediated endocytosis during vesicular stomatitis virus entry into host cells. Virology **338:**53–60.
32. **Timpe, J. M., Z. Stamataki, A. Jennings, K. Hu, M. J. Farquhar, H. J. Harris, A. Schwarz, I. Desombere, G. L. Roels, P. Balfe, and J. A. McKeating.** 2008. Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. Hepatology **47:**17–24.
33. **Tscherne, D. M., C. T. Jones, M. J. Evans, B. D. Lindenbach, J. A. McKeating, and C. M. Rice.** 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J. Virol. **80:** 1734–1741.
34. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
35. **Walters, R. W., P. Freimuth, T. O. Moninger, I. Ganske, J. Zabner, and M. J. Welsh.** 2002. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell **110:**789–799.
36. **Ye, L., X. Wang, S. Wang, G. Luo, Y. Wang, H. Liang, and W. Ho.** 2008. Centrifugal enhancement of hepatitis C virus infection of human hepatocytes. J. Virol. Methods **148:**161–165.
37. **Yu, A. S., K. M. McCarthy, S. A. Francis, J. M. McCormack, J. Lai, R. A. Rogers, R. D. Lynch, and E. E. Schneeberger.** 2005. Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells. Am. J. Physiol. Cell Physiol. **288:**C1231–C1241.

ANEXO 3

ANEXO 3

Interrelación entre polarización celular, metabolismo de lipoproteínas y entrada de HCV en la célula huésped.

World J Gastroenterol. 2011 (en prensa).

La identificación de claudina-1 y ocludina como co-receptores de HCV hizo pensar que las TJs pudieran desempeñar un papel en la infección por HCV, siendo la polaridad de los hepatocitos importante para dicho proceso. Sorprendentemente, los estudios llevados a cabo hasta la fecha parecen indicar que las TJs no sólo no participan en la infección, sino que actúan como una barrera impidiendo la entrada del virus en la célula. Además, se ha sugerido que la funcionalidad de claudina-1 y ocludina como co-receptores virales está asociada a su localización fuera de la estructura de la TJ. Sin embargo, estos trabajos no son concluyentes ya que las herramientas comúnmente empleadas para estudiar los mecanismos de la infección por HCV *in vitro* no reproducen de manera fidedigna el proceso real en cuanto a la composición de la partícula viral y las características de la célula huésped.

El sistema más utilizado para el estudio de HCV *in vitro* es la línea celular derivada de hepatoma Huh7, ya que es altamente permisiva para la infección, replicación y producción viral. No obstante, las considerables diferencias existentes entre estos cultivos celulares y los hepatocitos en el hígado, en términos de diferenciación y polaridad celular, hacen que los resultados obtenidos deban ser interpretados con cautela. Varios estudios han establecido una relación directa entre polaridad celular y la biosíntesis de lipoproteínas, que a su vez está íntimamente ligada al ensamblaje de la progenie viral en las células infectadas. Por otra parte, hay evidencias de que las partículas virales presentes tanto en el suero de los pacientes como en el sobrenadante de hepatocitos primarios humanos en cultivo son significativamente diferentes al HCV generado a partir de células Huh7. Por ello, parece razonable pensar que las características estructurales y funcionales de los viriones puedan depender en gran medida del fenotipo de las células productoras. Además, dado que los co-receptores de HCV claudina-1 y ocludina son proteínas asociadas a TJs, el empleo de células que carecen de la polaridad y las TJs típicas de los hepatocitos puede comprometer de forma importante la veracidad de los resultados obtenidos. Por ello, es necesario el desarrollo de sistemas *in vitro* que permitan el estudio de HCV en un contexto más real, para así poder establecer conclusiones más firmes que sirvan

como base para entender los mecanismos de la infección por HCV y diseñar nuevas estrategias antivirales con más garantías de éxito.

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v17.i10.1

World J Gastroenterol 2011 March 14; 17(10): 1-0000
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Interplay among cellular polarization, lipoprotein metabolism, and hepatitis C virus entry

Ignacio Benedicto, Francisca Molina-Jiménez, Ricardo Moreno-Otero, Manuel López-Cabrera, Pedro L Majano

Ignacio Benedicto, Francisca Molina-Jiménez, Manuel López-Cabrera, Pedro L Majano, Molecular Biology Unit, Hospital Universitario de la Princesa, Instituto de Investigación Sanitaria Princesa (IP), Madrid 28006, Spain
Ignacio Benedicto, Francisca Molina-Jiménez, Ricardo Moreno-Otero, Manuel López-Cabrera, Pedro L Majano, CIBERehd, Instituto de Salud Carlos Ⅲ, Madrid 28029, Spain
Ricardo Moreno-Otero, Liver Unit, Hospital Universitario de la Princesa, Instituto de Investigación Sanitaria Princesa (IP), Madrid 28006, Spain
Manuel López-Cabrera, Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Madrid 28049, Spain
Author contributions: Benedicto I and Majano PL conceived, designed, and wrote the manuscript; Molina-Jiménez F, Moreno-Otero R and López-Cabrera M contributed to the design and critical revision of the article.
Supported by CIBERehd to Moreno-Otero R, López-Cabrera M and Majano PL; SAF2007-61201 (Ministerio de Educación y Ciencia) to López-Cabrera M; CP03/0020 (Instituto de Salud Carlos Ⅲ), SAF2007-60667 (Ministerio de Educación y Ciencia) and PI10/00101 (Ministerio de Ciencia e Innovación, Instituto de Salud Carlos Ⅲ, FEDER) to Majano PL. Benedicto I was financially supported by CIBERehd and Molina-Jiménez F by Instituto de Salud Carlos Ⅲ and FIB Hospital de la Princesa
Correspondence to: Dr. Pedro L Majano, Molecular Biology Unit, Hospital Universitario de la Princesa, C/ Diego de León 62, 28006 Madrid, Spain. pmajano.hlpr@salud.madrid.org
Telephone: +34-91-5202334 Fax: +34-91-3093911
Received: October 21, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: March 14, 2011

Abstract

Hepatitis C virus (HCV) infects more than three million new individuals worldwide each year. In a high percentage of patients, acute infections become chronic, eventually progressing to fibrosis, cirrhosis, and hepatocellular carcinoma. Given the lack of effective prophylactic or therapeutic vaccines, and the limited sustained virological response rates to current therapies, new approaches are needed to prevent, control, and clear HCV infection. Entry into the host cell, being the first step of the viral cycle, is a potential target for the design of new antiviral compounds. Despite the recent discovery of the tight junction (TJ)-associated proteins claudin-1 and occludin as HCV co-receptors, which is an important step towards the understanding of HCV entry, the precise mechanisms are still largely unknown. In addition, increasing evidence indicates that tools that are broadly employed to study HCV infection do not accurately reflect the real process in terms of viral particle composition and host cell phenotype. Thus, systems that more closely mimic natural infection are urgently required to elucidate the mechanisms of HCV entry, which will in turn help to design antiviral strategies against this part of the infection process.

Key words: Cellular polarization; Tight junctions; Lipoprotein metabolism; Hepatitis C virus

Peer reviewers: A Mithat Bozdayi, MD, PhD, Hepatology Institute, Department of Gastroenterology, Ankara Medical Faculty, Ankara University, 06100 Cebeci Ankara, Turkey; Takashi Kojima, DVM., PhD, Department of Pathology, Sapporo Medical University School of Medicine, S.1, W.17, Chuo-ku, Sapporo 060-8556, Japan

Benedicto I, Molina-Jiménez F, Moreno-Otero R, López-Cabrera M, Majano PL. Interplay among cellular polarization, lipoprotein metabolism, and hepatitis C virus entry. *World J Gastroenterol* 2011; 17(10): 0000-0000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v17/i10/1.htm DOI: http://dx.doi.org/10.3748/wjg.v17.i10.1

INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, positive-polarity RNA virus that belongs to the *Flaviviridae* family and infects mainly hepatocytes[1]. The HCV genome encodes a polyprotein that is processed by host and viral proteases to yield

ten mature products, which include three structural proteins [the capsid protein (core) and two envelope glycoproteins (E1 and E2)], the p7 protein, and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The HCV particle consists of a nucleocapsid surrounded by a lipid bilayer harboring the two envelope glycoproteins, which heterodimerize and play a major role in HCV entry[1]. Cellular infection begins with the attachment of the viral particle to the host cell and, after interacting with cell surface molecules, the virus is subjected to clathrin-mediated endocytosis and its envelope is fused with the endosomal membrane, releasing the viral genome into the cytosol[1]. These steps involve a set of attachment factors and cellular co-receptors, including highly sulfated heparan sulfate, the low-density lipoprotein receptor (LDL-R), the scavenger receptor class B type I (SR-BI), the tetraspanin CD81, and the tight junction (TJ) proteins, claudin-1 and occludin[2-4]. These molecules are not exclusively present in hepatocytes; therefore, HCV hepatotropism may be determined by other factors, such as the absence of inhibitory proteins[5].

In early reports, the soluble E2 envelope protein was employed as an approach to search for host cell HCV binding factors[6,7]. However, the conformation and function of the soluble glycoprotein may differ considerably from HCV envelope-anchored E2. In addition, envelope-anchored E2 is associated with E1[8], which may have additional implications in terms of receptor recognition and binding. The study of HCV entry was boosted by the use of HCV pseudotyped particles (HCVpp)[9], providing an infection system exclusively relying on the envelope glycoproteins. Since the establishment of a cell culture-derived HCV (HCVcc)[10-12], it has become the most powerful tool for studying HCV because it reflects the complete viral cycle. The use of both HCVpp and HCVcc has been of crucial importance in the discovery of new receptors, and has enabled high-throughput assays to test molecules for their ability to inhibit HCV entry.

INHIBITION OF HCV ENTRY AS A THERAPEUTIC ALTERNATIVE

HCV infection is the most frequent cause of liver failure worldwide[13,14]. Current therapies based on PEGylated interferon-alpha and ribavirin often fail to clear the infection and present a wide spectrum of systemic side effects[15]; therefore, alternative therapeutic options need to be developed. Among the different steps of HCV cycle, viral entry could be considered as a clinical target, especially in the context of orthotopic liver transplantation, where allograft reinfection occurs within hours after reperfusion and is followed by an accelerated chronic disease progression[16]. To date, several molecules have been found to inhibit HCV entry. Matsumura *et al*[17] showed that phosphorothioate oligonucleotides, previously described as HIV entry inhibitors[18], blocked HCV entry *in vivo*. Other inhibitors of HIV entry, such as cyanovirin-N, have also proved effective[19]. Furthermore, serum amyloid A, an acute phase protein mainly produced by the liver in response to different stimuli, including infections, has been demonstrated to inhibit HCV entry[20,21]. Moreover, the milk thistle (*Silybum marianum*)-derived silymarin and its purified flavonolignans have been recently shown to inhibit HCV infection both *in vitro* and in non-responder patients[22,23], blocking viral entry and transmission[24]. Therefore, increasing evidence suggests that blocking the entry step of HCV infection may be a good therapeutic alternative.

The genetic diversity of HCV contributes to its evasion from the host immune response[25], challenging the development of effective vaccines and virus-targeted inhibitors[26-28]. Nonetheless, this problem could be overcome by developing antiviral strategies aimed at blocking essential host factors for viral infection. To this end, multiple strategies have been pursued to inhibit HCV entry at different levels, including viral attachment, post-binding events, and fusion with the endosomal membrane[4,29]. One of these approaches consists of interfering with the interaction between the viral particle and cell surface co-receptors by the use of glycosaminoglycans, natural ligands, recombinant proteins, or blocking antibodies (Table 1). Notably, it has been demonstrated that antibodies against CD81 can prevent HCV infection of human liver-uPA-SCID mice[16], probably by inhibiting the E2-CD81 binding process[6,16,30]. As a more realistic and economical alternative, small molecules with similar properties could be used instead of blocking antibodies. In search of these compounds, several high-throughput screenings have been performed recently to identify molecules with the ability to inhibit HCV infection at the entry step[31-33]. Importantly, the possible cytopathic effects of these inhibitors should be assayed prior to starting clinical trials and considering them as potential therapeutic options. Chockalingam et al. developed a cell protection screen where cytotoxicity and inhibition of infection were evaluated simultaneously[32]. As a more practical approach, Gastaminza et al. performed the screening with a set of drugs that had already been clinically approved[31].

The study of HCV infection and the search for inhibitory molecules are usually carried out with the use of HCVpp or HCVcc, and a highly permissive cell line, such as Huh7 and its derivatives. However, several conflicting results have arisen when attempting to validate the data in a more pathophysiologically relevant context (Table 1). A soluble form of CD81 was shown to prevent infection of Huh7.5 cells by HCVcc; however, it was not effective when primary human hepatocytes (PHH) were challenged with serum-derived virus[34]. Moreover, infection of hepatoma-derived cell lines with HCVpp and HCVcc does not seem to depend on LDL-R[35-37], whereas it has been demonstrated to participate in the infection of PHH with HCV from human plasma[38]. These facts stress the importance of being cautious with results obtained from HCV surrogates and cell lines, which should be validated *in vivo* whenever possible or at least in systems that more closely mimic real infection.

CELL POLARIZATION, TJ-ASSOCIATED PROTEINS, AND HCV ENTRY

In contrast to "simple" polarized cells, which present the

Table 1 Inhibition of infectivity by the blockade of hepatitis C virus co-receptors in different systems

		Host			Viral particle			Ref.
Co-receptor	Blocking agent	Cell lines	PHH	*In vivo*	HCVpp	HCVcc	Serum	
Heparan sulfate	Heparin	x			Y			[87]
		x				Y		[30,88,89]
			x[1]			Y		[90]
LDL-R	Anti-LDL-R	x			N			[36]
			x				Y	[38]
	Soluble LDL-R		x				Y	[38]
	LDLs/VLDLs	x			N			[9,37,91]
		x				N		[37]
			x				Y	[38]
CD81	Anti-CD81	x			Y			[9,36,92]
		x				Y		[30,88,89]
			x		Y			[9,92]
			x			Y		[34]
			x				Y	[34]
				x		Y		[16]
	CD81-LEL	x			Y			[9,36]
			x			Y		[34]
			x				N	[34]
	Knockdown	x			Y			[37]
		x				Y		[89]
			x				Y	[34]
SR-BI	Anti-SR-BI	x			N			[36]
		x			Y			[35,93]
		x				Y		[30,89]
	BLT-4	x			N/Y[2]			[91,93]
	ITX 5061/7650	x				Y		[94]
			x		Y			[94]
	oxLDLs	x			Y			[37]
		x				Y		[37]
	Knockdown	x			N/Y[2]			[37]
		x				Y		[89]
Claudin-1	Anti-claudin-1	x			Y			[30,56]
		x				Y		[30,56]
			x		Y			[56]
			x				Y	[56]
	Knockdown	x			Y			[42,43,95]
		x				Y		[42,43,95]
Occludin	Knockdown	x			Y			[43,44,45]
		x				Y		[43,44,45]

x: Experimental system employed; Y: Inhibition of infection; N: No inhibition of infection; [1]HCV-core immortalized PHH; [2]Only in the presence of high density lipoproteins. HCVpp: Hepatitis C virus pseudotyped particles; HCVcc: cell culture-derived hepatitis C virus.

typical epithelial columnar phenotype with individual basolateral and apical domains, hepatocytic polarity is very peculiar and complex[39]. The plasma membrane of polarized hepatocytes is divided into several basolateral and apical poles, the latter forming a continuous network of bile canaliculi (BC) into which bile is secreted[40]. BC are delimited by TJs, which maintain cell polarity by separating apical from basolateral domains and form the intercellular barrier between bile and blood[41]. Claudin-1 was the first TJ-associated protein to be described as a HCV co-receptor[42]. Soon after, occludin was also shown to participate in HCV entry[43-45]. Despite these discoveries clearly pointing to a role of TJs in HCV cell entry, recent works have reported conflicting data about how cell junctions and polarity influence HCV infection. Perturbation of cellular junctions by calcium depletion promotes opposing effects depending on the system employed, e.g. it decreases viral entry in Huh7 cells[46] but increases it in "simply" polarized Caco-2 cells[47]. Furthermore, junctional accumulation of claudin-1 has been shown to either improve[48] or hinder[49] infection of Huh7.5 and polarized HepG2 cells, respectively. Collectively, these data suggest that the HCV entry process may vary considerably depending on the polarization state of the target cells.

Several studies have questioned the importance of TJ integrity in the function of claudin-1 and occludin as HCV co-receptors. For example, HCV infection is not affected after knocking down other TJ-associated proteins, such as ZO-1 and JAM-A[45], and claudin-1's association with CD81 at the basolateral membrane of HepG2 cells, but not at the TJ, defines HCV entry[50,51]. Furthermore, fluorescent HCV particle internalization generally occurs outside of cell-cell junctions[52], and VEGF induces a reduction of junctional occludin concomitant with an increase of HCV infectiv-

ity[53]. Moreover, claudin-1 and occludin mutants lacking domains that are important for their correct junctional localization and function are still capable of rendering cells susceptible to HCVpp entry[42,54,55]. Finally, HCV infection of HepG2 cells is negatively regulated by cell polarity[49,53], but is not affected by TNF-α- and IFN-γ-mediated TJ disruption[49], and claudin-1 blocking antibodies inhibit HCV infection without perturbing TJs[30,56]. Taken together, these data strongly suggest that the role of claudin-1 and occludin in viral entry is relevant, but not necessarily when these proteins are part of functional TJs, which may indeed be a barrier for HCV infection. Interestingly, it has recently been demonstrated that hepatitis A virus infects HepG2-derived cells from the basolateral domain and that TJ-dependent polarization restricts infection[57].

The mechanisms by which claudin-1 and occludin participate in HCV entry have not been clearly established. In both cases, an extracellular loop of the protein has been shown to be indispensable for infection[42,44,55,58]. Several reports have shown that occludin precipitates with HCV E2 in infected, transfected, or replicon-containing cells[43,55,59], but its direct interaction with viral particles or envelope glycoproteins has not been demonstrated. Additionally, it has been shown that occludin interacts with dynamin Ⅱ, a well known regulator of endocytosis[55]. This observation, along with data obtained from cell-cell fusion experiments[45], suggests that occludin might participate in late steps of the HCV entry process. Interestingly, occludin endocytosis has been implicated in group B coxsackievirus infection, although not by directly interacting with the virus[60]. On the other hand, kinetic studies with blocking antibodies have shown that claudin-1 mediates an HCV entry step closely linked to CD81[30]. Indeed, it has been described that basolateral pools of claudin-1 are associated with CD81 in polarized HepG2 cells[50,51], and that disrupting this interaction, either by site directed mutagenesis or claudin-1 blocking antibodies, neutralizes HCV infection by reducing E2 association with the cell surface[30,51]. However, Cukierman *et al*[54] generated a mutant version of claudin-1 which, in spite of maintaining its interaction with CD81, no longer localized to cell-cell contacts and lost HCV receptor properties. This result suggests that, besides favoring E2 binding to the host cell, additional mechanisms involving claudin-1 participation in HCV entry may exist. Nevertheless, as these experiments were carried out in the non-hepatic, non-polarized HEK cell line, data should be carefully interpreted. This is a good example of how cell polarity may influence the results obtained, especially when studying features of TJ-associated proteins in a hepatocellular context.

LIPID METABOLISM AND HCV ENTRY

Cell polarization is crucial for the correct localization and function of TJ-associated proteins with HCV receptor activity, which could in turn be important for viral entry[41,61]. In addition, polarization may affect other steps of the HCV cycle, such as assembly and egress. Indeed, it has been shown that assembly of RNA enveloped viruses in MDCK cells is closely related to cell polarization[62]. It is also noteworthy that polarization is tightly linked to lipoprotein secretion[63,64], especially because some low density natural HCV particles have been found to be complexed with ApoB and/or ApoE-positive triglyceride-rich lipoproteins[65-67]. This association is believed to take place during viral egress[68] because HCVcc virions were found to be secreted in a manner that parallels the formation of VLDLs[69-71]. Thus, cell polarization may influence lipoprotein secretion, which is important for the generation of correctly assembled HCV progeny. Indeed, non-polarized Huh7-derived cells have been shown to be unable to secrete authentic, ApoB-containing VLDLs[72]. In addition, when HCVcc generated in these cells was subjected to isopycnic gradient ultracentrifugation, it was found to have higher average buoyant density than viral particles obtained from PHH[72]. Interestingly, the density profile of serum-derived viral particles obtained from HCVcc-infected animals was significantly lower than that of the initial HCVcc inoculum[73]. In both reports, specific infectivity of PHH and serum-derived HCV particles was shown to be greatly increased compared to standard HCVcc, and these properties were lost after passaging the virus again in Huh7.5 cells. These findings strongly suggest that HCVcc generated in Huh7-derived cells probably presents a defective lipoprotein association, which may in turn affect viral infection.

The lipoprotein composition and distribution within the viral particle may thus be important for the mechanisms underlying HCV entry[74]. Indeed, several reports have shown that lipoprotein lipase and hepatic triglyceride lipase alter both the physiological characteristics and the infectivity of HCV[75-77]. This notion is supported by the fact that LDL-R participates in the first steps of the entry process[38], and that viral particle density is inversely correlated to infectivity *in vivo*[66]. Additionally, the virus-host interaction could be affected in a context of a lipoprotein-defective viral particle because of changes in the exposure and accessibility of E2 to cellular co-receptors. In fact, this could explain the differences observed between HCVcc and serum-derived HCV infection in terms of inhibition by the soluble CD81 large extracellular loop[34]. Moreover, in contrast to serum-derived HCV[38], infection with HCVcc and HCVpp seems to be LDL-R independent[35-37], probably because of differences in lipoprotein composition.

Therefore, it is necessary to study HCV entry in systems that more accurately mimic the viral cycle in a pathophysiological context, from viral particle generation to target cell infection. The interplay between HCV and hepatocytes seems to be closely related to their particular phenotype (Figure 1); therefore, data obtained from models that do not reflect hepatocellular polarization and lipoprotein secretion should be cautiously interpreted.

TOWARDS A MORE PHYSIOLOGICALLY RELEVANT MODEL

The use of HCVpp and HCVcc has meant a great advance in HCV research. Their adaptability to high-throughput

analysis has enabled genome-wide screening for host proteins involved in the HCV cycle at different stages. In addition, they provide a valuable tool for testing the possible antiviral activity of large chemical libraries of compounds. However, it is important to bear in mind the limitations of these *in vitro* models, as both viral particles and target cells differ notably from serum-derived HCV and polarized hepatocytes, respectively. Thus, results obtained with HCVpp and HCVcc should be validated in systems that more closely mimic real HCV infection before establishing firm conclusions. To this end, significant work is being done by Molina and colleagues, who have already confirmed a role for LDL-R and CD81 in serum-derived HCV infection of PHH[34,38]. This model is considered an accurate *in vitro* system, as cells can be maintained in a differentiated phenotype to retain their polarity and drug metabolizing capacities. In addition, they can be infected with serum-derived HCV of any genotype and, in contrast to what is observed in hepatoma cell lines (e.g. Huh7 cells and derivatives), the innate immune response is fully preserved[78]. However, production of measurable titers of progeny virus in this system has not been achieved[79], indicating that the model fails to reflect the entire HCV cycle. Recently, it has been shown that HCVcc infection of PHH results in the robust production of infectious viral particles, which were in turn able to efficiently infect naïve PHH[72]. This primary cultured-derived HCV (termed HCVpc), compared to HCVcc, exhibited lower average buoyant density and higher specific infectivity, reminiscent of what is seen for virus recovered from the blood of animals infected with HCVcc[72,73]. Therefore, HCVpc infection of PHH emerges as a valuable tool for studying the complete HCV cycle in a more relevant context. Nevertheless, this system presents the drawbacks of working with PHH, such as restricted availability, the difficulty of studying long-term infections, and the heterogeneity of samples and results. Interestingly, Matrigel-embedded 3D cultures of Huh7 cells display a hepatocytic-like polarization and are susceptible to HCVcc infection. Progeny viruses generated by these cultures, similarly to HCVpc, also present a shift towards lower densities (our unpublished observations). This result suggests that if hepatocellular polarity is achieved, it is possible to generate viral particles that more closely mimic real HCV, even when cell lines are used as the source of virus.

Animal models are essential to validate *in vitro* data, because not only hepatocytes, but also the liver as a whole, may determine the mechanisms of HCV cell entry. Indeed, the liver sinusoidal endothelial cell-expressed protein, L-SIGN, has been shown to bind serum-derived HCV[80] and mediate transinfection of Huh7 cells by HCVpp[81,82]. Additionally, co-culture of PHH with liver sinusoidal endothelial cells significantly increases the expression of the HCV co-receptor LDL-R[83]. Regarding *in vivo* obtained data, CD81 is the only co-receptor that has been demonstrated to participate in HCV infection using the human liver-uPA-SCID mouse model[16]. This system, albeit constituting a useful tool, is limited by the fact that the animals lack a functional immune system. This may be important,

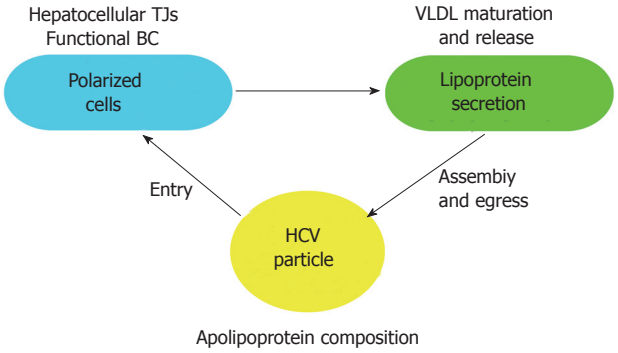


Figure 1 Interplay among cell polarization, lipoprotein secretion, and hepatitis C virus particle assembly, release, and entry into host cells. The correct polarization of hepatocytes, implying the presence of functional bile canaliculi delimited by TJs, may be important for the proper maturation and secretion of lipoproteins. This process is tightly associated with the composition and assembly of hepatitis C virus (HCV) lipoviroparticles and their exit from infected cells. Finally, HCV entry may be affected by the lipoprotein composition of the viral particle, the hepatocellular polarization of target cells, and the localization of the TJ-associated proteins claudin-1 and occludin.

not only for the outcome of the infection, but also for the entry process itself, because DC-SIGN, expressed on dendritic cells, has been shown to capture and transmit HCVpp to Huh7 cells[81,82]. More recently, peripheral blood B cells have been shown to exert a similar function[84]. To date, chimpanzees are the only immunocompetent *in vivo* system for studying HCV infection, but their use is limited by ethical concerns, restricted availability, requirement of special facilities, and very high costs[85]. In search of a small, HCV susceptible and immunocompetent animal model, it has been proposed to combine human liver chimeric models with mice harboring a human hematolymphoid system[85], although this approach depends on the availability of human primary cells. Thus, the ideal model would be an immunocompetent mouse susceptible to HCV infection without the need of harboring human cells. Given that HCV species tropism is restricted to human and primates[58,85], an alternative strategy consists of using an HCV variant able to infect murine cells. Bitzegeio et al. adapted HCVcc to mouse CD81 and identified three envelope glycoprotein mutations which together enhanced infection of cells with mouse or other rodent receptors by approximately 100-fold[86], thus overcoming the species-specific restriction of HCV cell entry. Another possible approach would be to employ genetically engineered mice bearing the human entry factors that confer species specificity[44]. However, mouse hepatocytes fail to initiate viral replication[85]; therefore, these models would not be able to mimic the entire HCV cycle.

CONCLUSION

During HCV infection, hepatocytes almost exclusively constitute both the target and the virus-producer cells. Thus, it is mandatory to perform HCV studies in systems that closely mimic the complex nature of hepatocytic pheno-

type. These models should enable the generation of viral particles that resemble the *in vivo* ones, and reproduce the hepatocyte physiology as accurately as possible. Only the combination of these two factors will provide the necessary information to establish firm conclusions. Nevertheless, the choice of employing HCVpp, HCVcc, HCVpc, or serum-derived HCV to infect cell lines, PHH, or animals should depend on the stage of the research process: the *in vitro* systems are more adequate for high throughput screenings and the *in vivo* models are essential for validating the data. Once the mechanisms of HCV entry are deciphered in detail, this step of the viral cycle could be an effective target for the development of antiviral compounds. These inhibitors should ideally be effective for a broad range of HCV genotypes and subtypes, and even for other viruses, such as HIV, that might share some entry mechanisms and co-infect some patients. Thus, blocking cellular factors might be a good therapeutic alternative in the fight against viral genetic variability. However, targeting host molecules could alter their physiological functions and result in harmful side effects. In addition, this approach does not rule out the possible emergence of viral variants that would be able to circumvent the specific effect of the entry inhibitor. Moreover, HCV cell-to-cell transmission may bypass the inhibition of cell-free virus entry and allow viral spread. Therefore, clinical strategies based on broad-spectrum compounds or the combination of different therapeutic molecules should be developed to simultaneously interfere with several steps of the viral cycle to efficiently control infection with the minimal side effects.

REFERENCES

1 **Lindenbach BD**, Rice CM. Unravelling hepatitis C virus replication from genome to function. *Nature* 2005; **436**: 933-938
2 **Dubuisson J**, Helle F, Cocquerel L. Early steps of the hepatitis C virus life cycle. *Cell Microbiol* 2008; **10**: 821-827
3 **Burlone ME**, Budkowska A. Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. *J Gen Virol* 2009; **90**: 1055-1070
4 **Zeisel MB**, Barth H, Schuster C, Baumert TF. Hepatitis C virus entry: molecular mechanisms and targets for antiviral therapy. *Front Biosci* 2009; **14**: 3274-3285
5 **Rocha-Perugini V**, Montpellier C, Delgrange D, Wychowski C, Helle F, Pillez A, Drobecq H, Le Naour F, Charrin S, Levy S, Rubinstein E, Dubuisson J, Cocquerel L. The CD81 partner EWI-2wint inhibits hepatitis C virus entry. *PLoS One* 2008; **3**: e1866
6 **Pileri P**, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938-941
7 **Scarselli E**, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 2002; **21**: 5017-5025
8 **Dubuisson J**. Hepatitis C virus proteins. *World J Gastroenterol* 2007; **13**: 2406-2415
9 **Bartosch B**, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 2003; **197**: 633-642
10 **Zhong J**, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294-9299
11 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796
12 **Lindenbach BD**, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626
13 **Poynard T**, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003; **362**: 2095-2100
14 **Pawlotsky JM**. Pathophysiology of hepatitis C virus infection and related liver disease. *Trends Microbiol* 2004; **12**: 96-102
15 **Feld JJ**, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* 2005; **436**: 967-972
16 **Meuleman P**, Hesselgesser J, Paulson M, Vanwolleghem T, Desombere I, Reiser H, Leroux-Roels G. Anti-CD81 antibodies can prevent a hepatitis C virus infection in vivo. *Hepatology* 2008; **48**: 1761-1768
17 **Matsumura T**, Hu Z, Kato T, Dreux M, Zhang YY, Imamura M, Hiraga N, Juteau JM, Cosset FL, Chayama K, Vaillant A, Liang TJ. Amphipathic DNA polymers inhibit hepatitis C virus infection by blocking viral entry. *Gastroenterology* 2009; **137**: 673-681
18 **Vaillant A**, Juteau JM, Lu H, Liu S, Lackman-Smith C, Ptak R, Jiang S. Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation. *Antimicrob Agents Chemother* 2006; **50**: 1393-1401
19 **Helle F**, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. *J Biol Chem* 2006; **281**: 25177-25183
20 **Lavie M**, Voisset C, Vu-Dac N, Zurawski V, Duverlie G, Wychowski C, Dubuisson J. Serum amyloid A has antiviral activity against hepatitis C virus by inhibiting virus entry in a cell culture system. *Hepatology* 2006; **44**: 1626-1634
21 **Cai Z**, Cai L, Jiang J, Chang KS, van der Westhuyzen DR, Luo G. Human serum amyloid A protein inhibits hepatitis C virus entry into cells. *J Virol* 2007; **81**: 6128-6133
22 **Polyak SJ**, Morishima C, Shuhart MC, Wang CC, Liu Y, Lee DY. Inhibition of T-cell inflammatory cytokines, hepatocyte NF-kappaB signaling, and HCV infection by standardized Silymarin. *Gastroenterology* 2007; **132**: 1925-1936
23 **Ferenci P**, Scherzer TM, Kerschner H, Rutter K, Beinhardt S, Hofer H, Schöniger-Hekele M, Holzmann H, Steindl-Munda P. Silibinin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. *Gastroenterology* 2008; **135**: 1561-1567
24 **Wagoner J**, Negash A, Kane OJ, Martinez LE, Nahmias Y, Bourne N, Owen DM, Grove J, Brimacombe C, McKeating JA, Pécheur EI, Graf TN, Oberlies NH, Lohmann V, Cao F, Tavis JE, Polyak SJ. Multiple effects of silymarin on the hepatitis C virus lifecycle. *Hepatology* 2010; **51**: 1912-1921
25 **Szabo G**, Dolganiuc A. HCV immunopathogenesis: virus-induced strategies against host immunity. *Clin Liver Dis* 2006; **10**: 753-771
26 **Stoll-Keller F**, Barth H, Fafi-Kremer S, Zeisel MB, Baumert TF. Development of hepatitis C virus vaccines: challenges and progress. *Expert Rev Vaccines* 2009; **8**: 333-345
27 **Zeisel MB**, Baumert TF. HCV entry and neutralizing antibodies: lessons from viral variants. *Future Microbiol* 2009; **4**: 511-517
28 **Georgel P**, Schuster C, Zeisel MB, Stoll-Keller F, Berg T, Bahram S, Baumert TF. Virus-host interactions in hepatitis C virus infection: implications for molecular pathogenesis and antiviral strategies. *Trends Mol Med* 2010; **16**: 277-286
29 **Barth H**, Liang TJ, Baumert TF. Hepatitis C virus entry: molecular biology and clinical implications. *Hepatology* 2006; **44**: 527-535
30 **Krieger SE**, Zeisel MB, Davis C, Thumann C, Harris HJ,

Schnober EK, Mee C, Soulier E, Royer C, Lambotin M, Grunert F, Dao Thi VL, Dreux M, Cosset FL, McKeating JA, Schuster C, Baumert TF. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. *Hepatology* 2010; **51**: 1144-1157
31 **Gastaminza P**, Whitten-Bauer C, Chisari FV. Unbiased probing of the entire hepatitis C virus life cycle identifies clinical compounds that target multiple aspects of the infection. *Proc Natl Acad Sci USA* 2010; **107**: 291-296
32 **Chockalingam K**, Simeon RL, Rice CM, Chen Z. A cell protection screen reveals potent inhibitors of multiple stages of the hepatitis C virus life cycle. *Proc Natl Acad Sci USA* 2010; **107**: 3764-3769
33 **Baldick CJ**, Wichroski MJ, Pendri A, Walsh AW, Fang J, Mazzucco CE, Pokornowski KA, Rose RE, Eggers BJ, Hsu M, Zhai W, Zhai G, Gerritz SW, Poss MA, Meanwell NA, Cockett MI, Tenney DJ. A novel small molecule inhibitor of hepatitis C virus entry. *PLoS Pathog* 2010; **6**, Epubahead of print
34 **Molina S**, Castet V, Pichard-Garcia L, Wychowski C, Meurs E, Pascussi JM, Sureau C, Fabre JM, Sacunha A, Larrey D, Dubuisson J, Coste J, McKeating J, Maurel P, Fournier-Wirth C. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent. *J Virol* 2008; **82**: 569-574
35 **Bartosch B**, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset FL. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* 2003; **278**: 41624-41630
36 **Hsu M**, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci USA* 2003; **100**: 7271-7276
37 **von Hahn T**, Lindenbach BD, Boullier A, Quehenberger O, Paulson M, Rice CM, McKeating JA. Oxidized low-density lipoprotein inhibits hepatitis C virus cell entry in human hepatoma cells. *Hepatology* 2006; **43**: 932-942
38 **Molina S**, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, Roitelman J, Barbaras R, Graber P, Ghersa P, Smolarsky M, Funaro A, Malavasi F, Larrey D, Coste J, Fabre JM, Sa-Cunha A, Maurel P. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol* 2007; **46**: 411-419
39 **Decaens C**, Durand M, Grosse B, Cassio D. Which in vitro models could be best used to study hepatocyte polarity? *Biol Cell* 2008; **100**: 387-398
40 **Easter DW**, Wade JB, Boyer JL. Structural integrity of hepatocyte tight junctions. *J Cell Biol* 1983; **96**: 745-749
41 **Lee NP**, Luk JM. Hepatic tight junctions: from viral entry to cancer metastasis. *World J Gastroenterol* 2010; **16**: 289-295
42 **Evans MJ**, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wölk B, Hatziioannou T, McKeating JA, Bieniasz PD, Rice CM. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. *Nature* 2007; **446**: 801-805
43 **Liu S**, Yang W, Shen L, Turner JR, Coyne CB, Wang T. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *J Virol* 2009; **83**: 2011-2014
44 **Ploss A**, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* 2009; **457**: 882-886
45 **Benedicto I**, Molina-Jiménez F, Bartosch B, Cosset FL, Lavillette D, Prieto J, Moreno-Otero R, Valenzuela-Fernández A, Aldabe R, López-Cabrera M, Majano PL. The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection. *J Virol* 2009; **83**: 8012-8020
46 **Brazzoli M**, Bianchi A, Filippini S, Weiner A, Zhu Q, Pizza M, Crotta S. CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. *J Virol* 2008; **82**: 8316-8329
47 **Mee CJ**, Grove J, Harris HJ, Hu K, Balfe P, McKeating JA. Effect of cell polarization on hepatitis C virus entry. *J Virol* 2008; **82**: 461-470
48 **Schwarz AK**, Grove J, Hu K, Mee CJ, Balfe P, McKeating JA. Hepatoma cell density promotes claudin-1 and scavenger receptor BI expression and hepatitis C virus internalization. *J Virol* 2009; **83**: 12407-12414
49 **Mee CJ**, Harris HJ, Farquhar MJ, Wilson G, Reynolds G, Davis C, van IJzendoorn SC, Balfe P, McKeating JA. Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells. *J Virol* 2009; **83**: 6211-6221
50 **Harris HJ**, Farquhar MJ, Mee CJ, Davis C, Reynolds GM, Jennings A, Hu K, Yuan F, Deng H, Hubscher SG, Han JH, Balfe P, McKeating JA. CD81 and claudin 1 coreceptor association: role in hepatitis C virus entry. *J Virol* 2008; **82**: 5007-5020
51 **Harris HJ**, Davis C, Mullins JG, Hu K, Goodall M, Farquhar MJ, Mee CJ, McCaffrey K, Young S, Drummer H, Balfe P, McKeating JA. Claudin association with CD81 defines hepatitis C virus entry. *J Biol Chem* 2010; **285**: 21092-21102
52 **Coller KE**, Berger KL, Heaton NS, Cooper JD, Yoon R, Randall G. RNA interference and single particle tracking analysis of hepatitis C virus endocytosis. *PLoS Pathog* 2009; **5**: e1000702
53 **Mee CJ**, Farquhar MJ, Harris HJ, Hu K, Ramma W, Ahmed A, Maurel P, Bicknell R, Balfe P, McKeating JA. Hepatitis C virus infection reduces hepatocellular polarity in a vascular endothelial growth factor-dependent manner. *Gastroenterology* 2010; **138**: 1134-1142
54 **Cukierman L**, Meertens L, Bertaux C, Kajumo F, Dragic T. Residues in a highly conserved claudin-1 motif are required for hepatitis C virus entry and mediate the formation of cell-cell contacts. *J Virol* 2009; **83**: 5477-5484
55 **Liu S**, Kuo W, Yang W, Liu W, Gibson GA, Dorko K, Watkins SC, Strom SC, Wang T. The second extracellular loop dictates Occludin-mediated HCV entry. *Virology* 2010; **407**: 160-170
56 **Fofana I**, Krieger SE, Grunert F, Glauben S, Xiao F, Fafi-Kremer S, Soulier E, Royer C, Thumann C, Mee CJ, McKeating JA, Dragic T, Pessaux P, Stoll-Keller F, Schuster C, Thompson J, Baumert TF. Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes. *Gastroenterology* 2010; **139**: 953-964, **964**.e1-e4
57 **Snooks MJ**, Bhat P, Mackenzie J, Counihan NA, Vaughan N, Anderson DA. Vectorial entry and release of hepatitis A virus in polarized human hepatocytes. *J Virol* 2008; **82**: 8733-8742
58 **Michta ML**, Hopcraft SE, Narbus CM, Kratovac Z, Israelow B, Sourisseau M, Evans MJ. Species-specific regions of occludin required by hepatitis C virus for cell entry. *J Virol* 2010; **84**: 11696-11708
59 **Benedicto I**, Molina-Jiménez F, Barreiro O, Maldonado-Rodríguez A, Prieto J, Moreno-Otero R, Aldabe R, López-Cabrera M, Majano PL. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. *Hepatology* 2008; **48**: 1044-1053
60 **Coyne CB**, Shen L, Turner JR, Bergelson JM. Coxsackievirus entry across epithelial tight junctions requires occludin and the small GTPases Rab34 and Rab5. *Cell Host Microbe* 2007; **2**: 181-192
61 **Perrault M**, Pécheur EI. The hepatitis C virus and its hepatic environment: a toxic but finely tuned partnership. *Biochem J* 2009; **423**: 303-314
62 **Rodriguez-Boulan E**, Paskiet KT, Sabatini DD. Assembly of enveloped viruses in Madin-Darby canine kidney cells: polarized budding from single attached cells and from clusters of cells in suspension. *J Cell Biol* 1983; **96**: 866-874
63 **Traber MG**, Kayden HJ, Rindler MJ. Polarized secretion of

newly synthesized lipoproteins by the Caco-2 human intestinal cell line. *J Lipid Res* 1987; **28**: 1350-1363
64 **Ratcliffe DR**, Iqbal J, Hussain MM, Cramer EB. Fibrillar collagen type I stimulation of apolipoprotein B secretion in Caco-2 cells is mediated by beta1 integrin. *Biochim Biophys Acta* 2009; **1791**: 1144-1154
65 **André P**, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, Pol S, Bréchot C, Paranhos-Baccalà G, Lotteau V. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol* 2002; **76**: 6919-6928
66 **André P**, Perlemuter G, Budkowska A, Bréchot C, Lotteau V. Hepatitis C virus particles and lipoprotein metabolism. *Semin Liver Dis* 2005; **25**: 93-104
67 **Diaz O**, Delers F, Maynard M, Demignot S, Zoulim F, Chambaz J, Trépo C, Lotteau V, André P. Preferential association of Hepatitis C virus with apolipoprotein B48-containing lipoproteins. *J Gen Virol* 2006; **87**: 2983-2991
68 **Gastaminza P**, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J Virol* 2008; **82**: 2120-2129
69 **Chang KS**, Jiang J, Cai Z, Luo G. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. *J Virol* 2007; **81**: 13783-13793
70 **Gastaminza P**, Kapadia SB, Chisari FV. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. *J Virol* 2006; **80**: 11074-11081
71 **Meunier JC**, Russell RS, Engle RE, Faulk KN, Purcell RH, Emerson SU. Apolipoprotein c1 association with hepatitis C virus. *J Virol* 2008; **82**: 9647-9656
72 **Podevin P**, Carpentier A, Pène V, Aoudjehane L, Carrière M, Zaïdi S, Hernandez C, Calle V, Méritet JF, Scatton O, Dreux M, Cosset FL, Wakita T, Bartenschlager R, Demignot S, Conti F, Rosenberg AR, Calmus Y. Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. *Gastroenterology* 2010; **139**: 1355-1364
73 **Lindenbach BD**, Meuleman P, Ploss A, Vanwolleghem T, Syder AJ, McKeating JA, Lanford RE, Feinstone SM, Major ME, Leroux-Roels G, Rice CM. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci USA* 2006; **103**: 3805-3809
74 **Popescu CI**, Dubuisson J. Role of lipid metabolism in hepatitis C virus assembly and entry. *Biol Cell* 2009; **102**: 63-74
75 **Thomssen R**, Bonk S. Virolytic action of lipoprotein lipase on hepatitis C virus in human sera. *Med Microbiol Immunol* 2002; **191**: 17-24
76 **Andréo U**, Maillard P, Kalinina O, Walic M, Meurs E, Martinot M, Marcellin P, Budkowska A. Lipoprotein lipase mediates hepatitis C virus (HCV) cell entry and inhibits HCV infection. *Cell Microbiol* 2007; **9**: 2445-2456
77 **Shimizu Y**, Hishiki T, Sugiyama K, Ogawa K, Funami K, Kato A, Ohsaki Y, Fujimoto T, Takaku H, Shimotohno K. Lipoprotein lipase and hepatic triglyceride lipase reduce the infectivity of hepatitis C virus (HCV) through their catalytic activities on HCV-associated lipoproteins. *Virology* 2010; **407**: 152-159
78 **Gondeau C**, Pichard-Garcia L, Maurel P. Cellular models for the screening and development of anti-hepatitis C virus agents. *Pharmacol Ther* 2009; **124**: 1-22
79 **Buck M**. Direct infection and replication of naturally occurring hepatitis C virus genotypes 1, 2, 3 and 4 in normal human hepatocyte cultures. *PLoS One* 2008; **3**: e2660
80 **Gardner JP**, Durso RJ, Arrigale RR, Donovan GP, Maddon PJ, Dragic T, Olson WC. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. *Proc Natl Acad Sci USA* 2003; **100**: 4498-4503
81 **Lozach PY**, Amara A, Bartosch B, Virelizier JL, Arenzana-Seisdedos F, Cosset FL, Altmeyer R. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem* 2004; **279**: 32035-32045
82 **Cormier EG**, Durso RJ, Tsamis F, Boussemart L, Manix C, Olson WC, Gardner JP, Dragic T. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. *Proc Natl Acad Sci USA* 2004; **101**: 14067-14072
83 **Nahmias Y**, Casali M, Barbe L, Berthiaume F, Yarmush ML. Liver endothelial cells promote LDL-R expression and the uptake of HCV-like particles in primary rat and human hepatocytes. *Hepatology* 2006; **43**: 257-265
84 **Stamataki Z**, Shannon-Lowe C, Shaw J, Mutimer D, Rickinson AB, Gordon J, Adams DH, Balfe P, McKeating JA. Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells. *Blood* 2009; **113**: 585-593
85 **Ploss A**, Rice CM. Towards a small animal model for hepatitis C. *EMBO Rep* 2009; **10**: 1220-1227
86 **Bitzegeio J**, Bankwitz D, Hueging K, Haid S, Brohm C, Zeisel MB, Herrmann E, Iken M, Ott M, Baumert TF, Pietschmann T. Adaptation of hepatitis C virus to mouse CD81 permits infection of mouse cells in the absence of human entry factors. *PLoS Pathog* 2010; **6**: e1000978
87 **Barth H**, Schnober EK, Zhang F, Linhardt RJ, Depla E, Boson B, Cosset FL, Patel AH, Blum HE, Baumert TF. Viral and cellular determinants of the hepatitis C virus envelope-heparan sulfate interaction. *J Virol* 2006; **80**: 10579-10590
88 **Koutsoudakis G**, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, Bartenschlager R. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. *J Virol* 2006; **80**: 5308-5320
89 **Zeisel MB**, Koutsoudakis G, Schnober EK, Haberstroh A, Blum HE, Cosset FL, Wakita T, Jaeck D, Doffoel M, Royer C, Soulier E, Schvoerer E, Schuster C, Stoll-Keller F, Bartenschlager R, Pietschmann T, Barth H, Baumert TF. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. *Hepatology* 2007; **46**: 1722-1731
90 **Basu A**, Kanda T, Beyene A, Saito K, Meyer K, Ray R. Sulfated homologues of heparin inhibit hepatitis C virus entry into mammalian cells. *J Virol* 2007; **81**: 3933-3941
91 **Voisset C**, Callens N, Blanchard E, Op De Beeck A, Dubuisson J, Vu-Dac N. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. *J Biol Chem* 2005; **280**: 7793-7799
92 **Cormier EG**, Tsamis F, Kajumo F, Durso RJ, Gardner JP, Dragic T. CD81 is an entry coreceptor for hepatitis C virus. *Proc Natl Acad Sci USA* 2004; **101**: 7270-7274
93 **Bartosch B**, Verney G, Dreux M, Donot P, Morice Y, Penin F, Pawlotsky JM, Lavillette D, Cosset FL. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. *J Virol* 2005; **79**: 8217-8229
94 **Syder AJ**, Lee H, Zeisel MB, Grove J, Soulier E, Macdonald J, Chow S, Chang J, Baumert TF, McKeating JA, McKelvy J, Wong-Staal F. Small molecule scavenger receptor BI antagonists are potent HCV entry inhibitors. *J Hepatol* 2011; **54**: 48-55
95 **Meertens L**, Bertaux C, Cukierman L, Cormier E, Lavillette D, Cosset FL, Dragic T. The tight junction proteins claudin-1, -6, and -9 are entry cofactors for hepatitis C virus. *J Virol* 2008; **82**: 3555-3560

S- Editor Sun H **L- Editor** Stewart GJ **E- Editor** Ma WH

ANEXO 4

ANEXO 4

Cultivos celulares incluidos en Matrigel como modelo para estudiar la infección por HCV en un contexto altamente polarizado

Sometido

Los sistemas derivados de PHHs son considerados como los mejores modelos *in vitro* para el estudio del ciclo infectivo de HCV, ya que las células se pueden mantener con un fenotipo diferenciado y preservan sus características metabólicas. Sin embargo, el uso de PHHs presenta varios inconvenientes, como son su limitada disponibilidad y su gran variabilidad interindividual, lo que dificulta la estandarización de los procedimientos de aislamiento y los resultados obtenidos. Por ello, es necesario el desarrollo de sistemas que estén basados en líneas celulares pero que a su vez se asemejen a los hepatocitos en su entorno natural.

Con este propósito se realizaron cultivos tridimensionales (3D) de líneas celulares derivadas de hepatocitos humanos mediante su inclusión en Matrigel, un producto comercial compuesto por una mezcla de proteínas de la matriz extracelular. Se observó que, a diferencia de los cultivos bidimensionales estándar, en estas condiciones las células adquirían la polarización típica de los hepatocitos y formaban estructuras funcionales semejantes a los canalículos biliares, estando delimitadas por proteínas asociadas a TJs. Además, se comprobó que estos cultivos eran susceptibles de ser infectados por HCV y tenían la capacidad de producir nuevas partículas virales infectivas. Por último, se pudo observar que el virus generado en 3D era más parecido al existente en pacientes en términos de densidad e infectividad específica, en comparación con el virus obtenido en condiciones de cultivo tradicionales. En resumen, este sistema constituye un modelo para el estudio del ciclo completo de HCV en un contexto más relevante que el proporcionado por el cultivo tradicional de líneas celulares. Además, gracias a su versatilidad y adaptabilidad a análisis “high-throughput”, puede constituir una herramienta importante para la búsqueda y evaluación de compuestos antivirales.

Matrigel-Embedded 3D Culture of Hepatoma-Derived Cell Lines as a Hepatocyte-Like Polarized System to Study Hepatitis C Virus Cycle.

Sometido

Francisca Molina-Jimenez*, Ignacio Benedicto*, Viet Loan Dao Thi, Virginia Gondar, Dimitri Lavillette, Jose J. G. Marin, Oscar Briz, Ricardo Moreno-Otero, Rafael Aldabe, Thomas F. Baumert, François-Loïc Cosset, Manuel Lopez-Cabrera, and Pedro L. Majano

*These authors contributed equally to this work.

ABSTRACT

Background & Aims: Hepatocytes are highly polarized cells where intercellular junctions, including tight junctions (TJs), determine the polarity. Recently, the TJ-associated proteins claudin-1 and occludin have been implicated in hepatitis C virus (HCV) entry and spread. Nevertheless, cell line-based experimental systems that exhibit hepatocyte-like polarity and permit robust infection and virion production are not currently available, limiting the progress towards understanding the possible role of hepatocye polarity in HCV cycle. Thus, we sought to determine whether Matrigel-derived cultures could be used to study both HCV infection and viral production in a context of hepatocyte-like polarized cells. ***Methods:*** We developed Matrigel-embedded three dimensional (3D) cultures derived from human hepatoma cell lines and assessed the localization of apical and basolateral markers and the export of fluorescently labelled molecules. We tested the ability of these cultures to support HCV infection and generate new infective viral particles, which were characterized by ultracentrifugation through iodixanol gradients. ***Results:*** In contrast to standard bidimensional cultures, Matrigel-embedded cells adopted hepatocyte polarization features forming a continuous network of functional proto-bile canaliculi structures. These 3D cultures supported HCV infection by JFH-1 virus and produced viral particles whose infectivity depended on the expression of CD81, claudin-1 and occludin in 3D-cultured target cells. Interestingly, 3D-generated HCV particles shifted towards lower densities that exhibited a higher associated specific infectivity, resembling HCV virions present in natural infection. ***Conclusions:*** our findings describe a novel use for Matrigel-embedded, cell line-based cultures to study the entire HCV cycle in a more relevant context.

Key words: HCV infection, three dimensional cultures, tight junctions, cellular polarization, bile canaliculi.

Abbreviations: HCV, hepatitis C virus; SR-BI, scavenger receptor class B type I; TJ(s), tight junction(s); BC, bile canaliculi; ZO-1, zonula occludens protein-1; HCVcc, cell culture derived HCV; CMFDA, 5-chloromethylfluorescein di-acetate; CGamF, cholylglycylamido fluorescein; shRNA, short hairpin RNA; MRP2, multidrug resistance-associated protein 2; PHH, primary human hepatocytes; VLDLs, very low density lipoproteins.

INTRODUCTION

In a high percentage of patients, acute hepatitis C virus (HCV) infections become chronic and ultimately progress to fibrosis, cirrhosis and hepatocellular carcinoma [1]. The current therapy consisting of a combination of pegylated interferon-alpha plus ribavirin is limited by a response rate of less than 50% in patients infected with genotype 1 [2]. HCV, belonging to the *Flaviviridae* family, is a small enveloped RNA virus mainly transmitted by blood and other body fluids [3]. In plasma, HCV RNA-containing particles can be found associated with lipoproteins, which have been suggested to play a role in early steps of HCV infection [4]. Multiple evidence exists for the involvement of host cell factors in HCV entry, including glycosaminoglycans, the low-density lipoprotein receptor, scavenger receptor class B type I (SR-BI), the tetraspanin CD81 and the tight junction (TJ) proteins claudin-1 and occludin [5].

The correct functioning of the liver is ensured by the setting and maintenance of the highly polarized phenotype of hepatocytes. In contact with the external environment, the apical poles of front-facing and adjacent hepatocytes form a continuous network of bile canaliculi (BC) where bile is secreted [6]. TJs maintain cell polarity separating apical from basolateral membrane domains and form a paracellular barrier that allows the selective passage of certain solutes [7]. In hepatocytes, TJs seal the bile canaliculi and form the intercellular barrier between bile and blood [6]. Human hepatocyte-derived cell lines susceptible to HCV infection such as Huh7 and PLC/PRF/5 barely form BC structures, but TJ-associated proteins such as zonula occludens protein-1 (ZO-1), occludin and claudin-1 form belts at the cell apex as in ‘simple’ polarized cells [8, 9]. Thus, although TJ-associated proteins in these systems exert, at least to some extent, their typical functions (i. e., separate apical from basolateral domains and constitute a paracellular barrier), cellular models displaying hepatocyte-like polarity are needed to study HCV life cycle in a more physiologically relevant context [10, 11].

To date, it is not clearly established how hepatocyte polarity influences HCV cycle [10]. Several attempts have been carried out to reproduce the *in vivo* phenotype of hepatocytes in 3D-cultured cell lines and propagate HCV by using different kinds of bioreactors [12-14]. Despite cells were susceptible to infection and produced new infectious virions, no evidence was found to support the polarized localization of basolateral and apical markers or the existence of functional BC-like structures. Furthermore, HepG2 cells ectopically expressing CD81 have been used as a model of polarized culture to study HCV infection [15], but their restricted efficiency to support

infection (more than 700-fold reduction compared to Huh7.5 cells) [15, 16] limits their use as a robust system to study HCV assembly and egress. Herein, we describe that Matrigel-embedded cultures of human hepatoma-derived cell lines can be used as a 3D hepatocyte-like polarized system to study the entire HCV cycle.

MATERIALS AND METHODS

Cell culture, generation of HCV replicon-containing clones and HCVcc infection

For 3D Matrigel-embedded cultures, 50 µl of complete medium containing 5×10^3 cells was added to 50 µl of Growth factor-reduced Matrigel (BD Biosciences, San Jose, CA), gently mixed and deposited into either a 8-well chambered coverglass (Nalge Nunc International, Rochester, NY) or a 48-well plate (Corning Incorporated, Corning, NY), incubated at 37°C for 30 minutes and covered with complete medium. Huh7 cells expressing full-length or subgenomic HCV replicons were established as previously described [8]. JFH1 HCVcc was produced as previously described [8] and expanded in culture for several passages. For HCVcc infection assays, 5×10^3 cells grown on 2D (24-well plates) or 3D (48-well plates) cultures for 6 days were infected with 10^3 FFUs and processed as indicated.

Immunofluorescence analysis and confocal microscopy

Cells grown in chambered coverglasses were processed essentially as previously described [8] with minor modifications, employing the indicated antibodies (see Supplementary Materials and Methods section).

Western blots

Cells grown on 48-well plates for 3D Matrigel cultures or 24-well plates for 2D conventional cultures were lysed on the plate with 100 µl of Laemmli buffer and boiled for 5 minutes. Western blots were carried out as previously described [8].

Characterization of hepatocyte-like polarity

Functional BC, those that exhibited an accumulation of 5-chloromethylfluorescein di-acetate (CMFDA) or cholylglycylamido fluorescein (CGamF), were determined as previously described (see Supplementary Materials and Methods section). Cells were grown in chambered coverglasses with or without Matrigel for 6 days and incubated with 5 µM of either CMFDA or CGamF. Cells were rinsed and the accumulation of fluorescent products was analyzed by confocal analysis.

RT-PCR

RNA extraction, reverse transcription and quantitative PCR were performed as previously described [8]. Specific primers used are listed in the Supplementary Materials and Methods section.

Short hairpin RNA (shRNA) retroviral transfer.

Generation of control and occludin shRNA retroviral vectors, production of retroviral particles and infection was performed as previously described [9]. 5×10^3 transduced cells were reseeded in 2D (24-well plates) or 3D (48-well plates) cultures 8 h after the last transduction. Cells were grown for 6 days and either infected with HCVcc or lysed to check knockdown efficiency by Western blot.

Iodixanol gradient ultracentrifugation of 2D and 3D-generated HCVcc

2D and 3D-generated viral supernatants were subjected to ultracentrifugation through continuous 0-40% iodixanol gradients prepared using Optiprep (Axis-Shield, Oslo, Norway). Fraction titration and HCV RNA quantification were performed as described in the Supplementary Materials and Methods section.

RESULTS

Characterization of 3D cultures of human hepatocyte-derived cell lines.

Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells that resembles the complex extracellular environment found in many tissues and is used to produce thick 3D gels for cell culture [17]. It was observed that Matrigel-cultured Huh7 cells assembled into 3D spheroids, whereas standard 2D-cultured cells formed the typical epithelial monolayer (Fig. 1A). In 2D cultures, Na^+, K^+-ATPase was distributed basolaterally and radixin mainly localized to the apical pole of cells, resembling a 'simple' epithelial phenotype (Fig. 1B, top). In 3D cultures, both markers were also mutually exclusive; however, spheroids presented a canalicular-like radixin staining localized at membranes surrounding the lumens within 3D aggregates (Fig. 1B, bottom). Furthermore, in 2D cultures ZO-1 was confined to cell-cell junctions as in cells with 'simple' epithelial polarization, and the Golgi apparatus did not show any specific orientation within cells (Fig. 1C, top). In contrast, and similarly to polarized hepatocytes, the Golgi apparatus of spheroid-forming cells was clearly positioned between the nuclei and the lumen, whose edges were positive for ZO-1 (Fig. 1C, bottom). Overall, these data suggested that 3D-cultures presented hepatocyte-like polarization and developed TJ-delimited canalicular structures.

Hepatocyte apical domain is designed for the secretion of bile into BC [6]. Among other proteins, radixin is a critical requirement for the normal maintenance of the canalicular membrane and the localization and function of its transport proteins [18]. As shown in Fig. 1B, radixin localized around the lumen of spheroids, suggesting that the apical membranes might behave as functional bile secretion domains. To further investigate the functionality of these BC-like structures, we next analyzed the expression levels and subcellular localization of the multidrug resistance protein 2 (MRP2), essential for the secretory function of differentiated hepatocytes. We analyzed MRP2 mRNA and protein levels and observed no evident differences between 2D and 3D cultures (Fig. 2A and data not shown). However, whereas in 2D cultures MRP2 was not concentrated at any particular localization, 3D spheroids presented a marked accumulation of MRP2 at the membranes delimiting the BC-like structure (Fig. 2B). Furthermore, to analyze the cell export ability of 2D and 3D cultures, they were loaded with CMFDA (a general cell stain) or CGamF (a conjugated bile acid analog) and visualized by confocal microscopy. 2D-cultured cells were not able to secrete the compounds, as evidenced

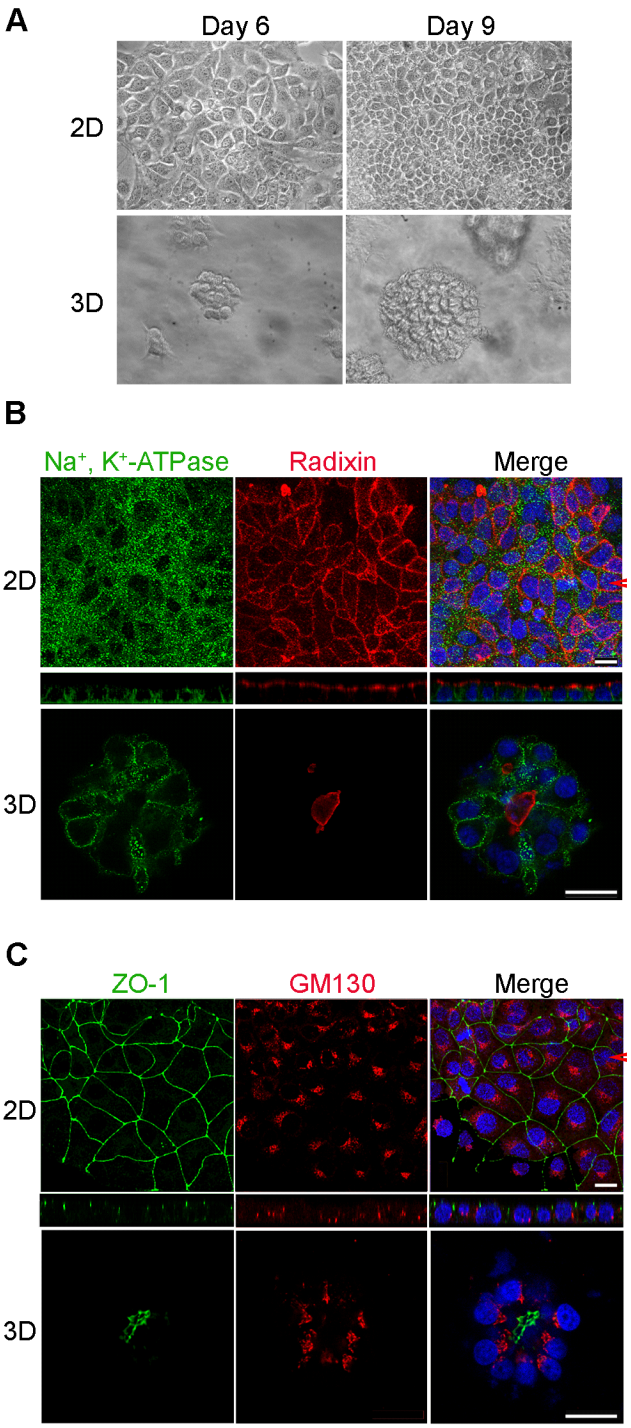


Fig. 1. Characterization of Matrigel-embedded 3D aggregates of the human hepatocyte-derived cell line Huh7. (A) Phase contrast image (20x magnification) of 2D and 3D Huh7 cultures 6 and 9 days after seeding. **(B and C)** Cells were grown in 2D and 3D conditions for 6 days and processed for double-label immunofluorescence and confocal analysis. Na^+, K^+-ATPase (basolateral marker) and ZO-1, green; Radixin (apical marker) and GM130 (a Golgi matrix protein), red; nuclei were stained with DAPI (blue). 2D images show the merged projection of confocal stacks (top) and X-Z sections (bottom). Arrows indicate the plane from which the Z sections were taken. 3D images show a single X-Y section from the confocal stacks. Z sections were compiled by taking 0.5 μm steps through each X-Y section. Photographs are representative of at least three separate experiments. Bar, 25 μm.

by a clear intracellular retention (Fig. 2C). In contrast, spheroid-forming cells efficiently exported the labelled molecules to the canalicular space where they accumulated (Fig. 2C). These data strongly suggested that, in contrast to standard 2D cultures, 3D spheroids developed BC-like structures that presented vectorial trafficking of compounds. In summary, 3D-cultured Huh7 cells displayed structural and functional polarity feautures similar to hepatocytes *in vivo*. Data presented in Fig. 1 and Fig. 2 were confirmed with the hepatocyte-derived cell line PLC/PRF/5 (Supplementary Figs.

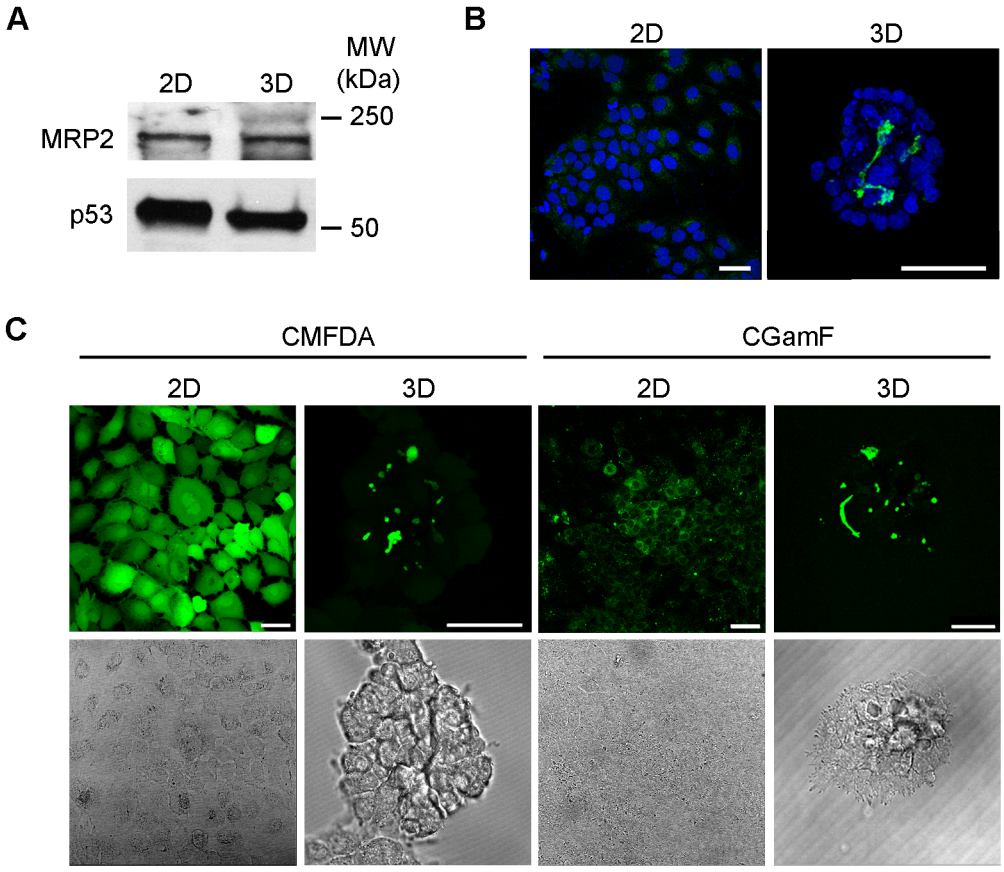


Fig. 2. BC-like structure and function in 2D and 3D-cultured Huh7 cells. (A) MRP2 protein levels were analyzed by Western blot. p53 was used as loading control. Molecular weight markers (kDa) are indicated on the right. **(B)** MRP2 localization after 6 days of 2D and 3D culture was analyzed by confocal immunofluorescence analysis. Images show the merged projection of confocal stacks. MRP2, green; nuclei, blue. Bar, 50 μm. **(C)** Cells were 2D or 3D cultured for 6 days, treated with 5 μM CMFDA or CGamF and washed. Confocal images show the accumulation of CMFDA and CGamF. Bar, 50 μm. Results shown are representative of at least three separate experiments.

S1 and S2). In order to study whether the differentiation of Huh7 cells was improved in 3D cultures, we analyzed the expression of hepatocyte-specific markers by real-time RT-PCR and no major changes were observed (Supplementary Fig. S3).

3D cultures are susceptible to HCV infection.

Firstly, we compared the expression levels of the HCV (co)receptors SR-BI, CD81, occludin and claudin-1 between 2D and 3D cultures by Western blot analysis, and no substantial differences were found (Fig. 3A). We next analyzed whether the localization

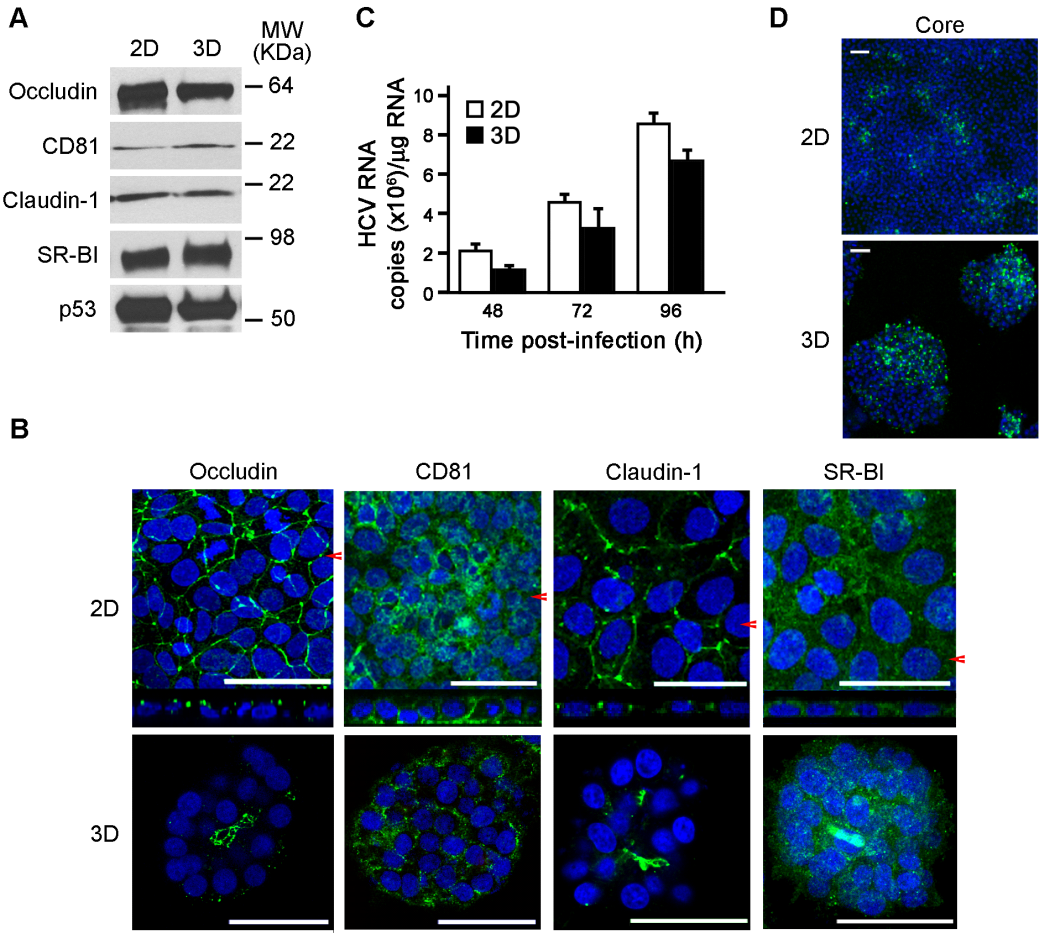


Fig. 3. 3D-cultured Huh7 cells are susceptible to HCV infection. (A) The expression of HCV (co)receptors (occludin, CD81, claudin-1 and SR-BI) was analyzed by Western blot after 6 days of 2D or 3D culture. **(B)** The localization of HCV (co)receptors was analyzed by immunofluorescence and confocal analysis. Images show the merged projection of confocal stacks. Arrows indicate the planes corresponding to the X-Z sections shown (2D, bottom). Nuclei were stained with DAPI (blue). Images are representative of three independent experiments. Bar, 50 µm. **(C)** Cells were grown in 2D or 3D conditions for 6 days and infected overnight with 2D-produced HCVcc. Cells were cultured for further 48, 72 or 96 h, total RNA was extracted and HCV RNA was quantified by real-time RT-PCR. Data are represented as the mean value ± SD of a triplicate experiment. **(D)** Cells were cultured in 2D or 3D conditions for 6 days and infected with HCVcc. Three days after infection, cells were processed for immunostaining using anti-core specific Ab (green). Nuclei were stained with DAPI (blue). Images show the merged projection of confocal stacks. Bar, 50 µm. Results are representative of two separate experiments.

of the HCV (co)receptors in 3D cultures was altered when compared to 2D cultures. Similar to previous studies [8, 9], staining of occludin and claudin-1 in 2D cultures appeared to localize at the apical poles of lateral cell membranes, the typical

distribution of these proteins in cells with 'simple' epithelial polarization (Fig. 3B). However, in 3D cultures occludin and claudin-1 seemed to present a similar localization as ZO-1 (Fig. 1C), being mainly detected surrounding the BC-like structures. These results are in agreement with the localization of claudin-1 in polarized HepG2 cells [19]. Additionally, results showed that CD81 and SR-BI appeared diffusely distributed in both 2D and 3D cultures (Fig. 3B). These data indicated that embedding Huh7 cells in Matrigel did not significantly affect the expression levels of the HCV (co)receptors analyzed but altered the spatial distribution of occludin and claudin-1, which were concentrated at hepatocytic-like TJ structures. Nevertheless, we cannot rule out a possible extrajunctional, diffuse localization of a pool of TJ-associated proteins, which has been already described in other polarized systems [19].

We next sought to determine whether 3D cultures were susceptible to HCV infection. We performed infection assays using HCVcc and 2D or 3D-cultured Huh7 cells , and monitored viral RNA at different time points post-infection by real-time RT-PCR (Fig. 3C). We also included controls without cells in both 2D and 3D conditions (data not shown). HCV RNA was only detected when cells were present, demonstrating that the signal obtained was not due to the amplification of Matrigel-bound input HCVcc RNA. Additionally, viral RNA levels increased with time, strongly suggesting that productive infection and active replication was taking place. In order to compare HCV replication in 2D and 3D cultures, HCV replicon-containing cells were cultured in both conditions and viral RNA was measured by real-time RT-PCR. No differences were observed between 2D and 3D cultures (Supplementary Fig. S4). Therefore, these data indicated that 3D-cultured cells were infected by HCVcc to a similar extent as 2D cultures, and that viral RNA replication was not affected by embedding cells in Matrigel. To further confirm the susceptibility of spheroids to be infected, we performed immunofluorescence analysis of the viral core protein in mock or HCVcc-infected Huh7 cells cultured in 2D or 3D conditions (Fig. 3D and Supplementary Fig. S5). We clearly observed viral protein-positive cells, demonstrating that 3D cultures were permissive for HCVcc infection. Similar results were obtained in PLC/PRF/5 cells (Supplementary Fig. S6).

Characterization of HCVcc produced by 3D-cultured cells.

We then investigated the possible release of infectious virions from HCVcc-infected spheroids. We found that supernatants derived from HCVcc-infected Huh7 and

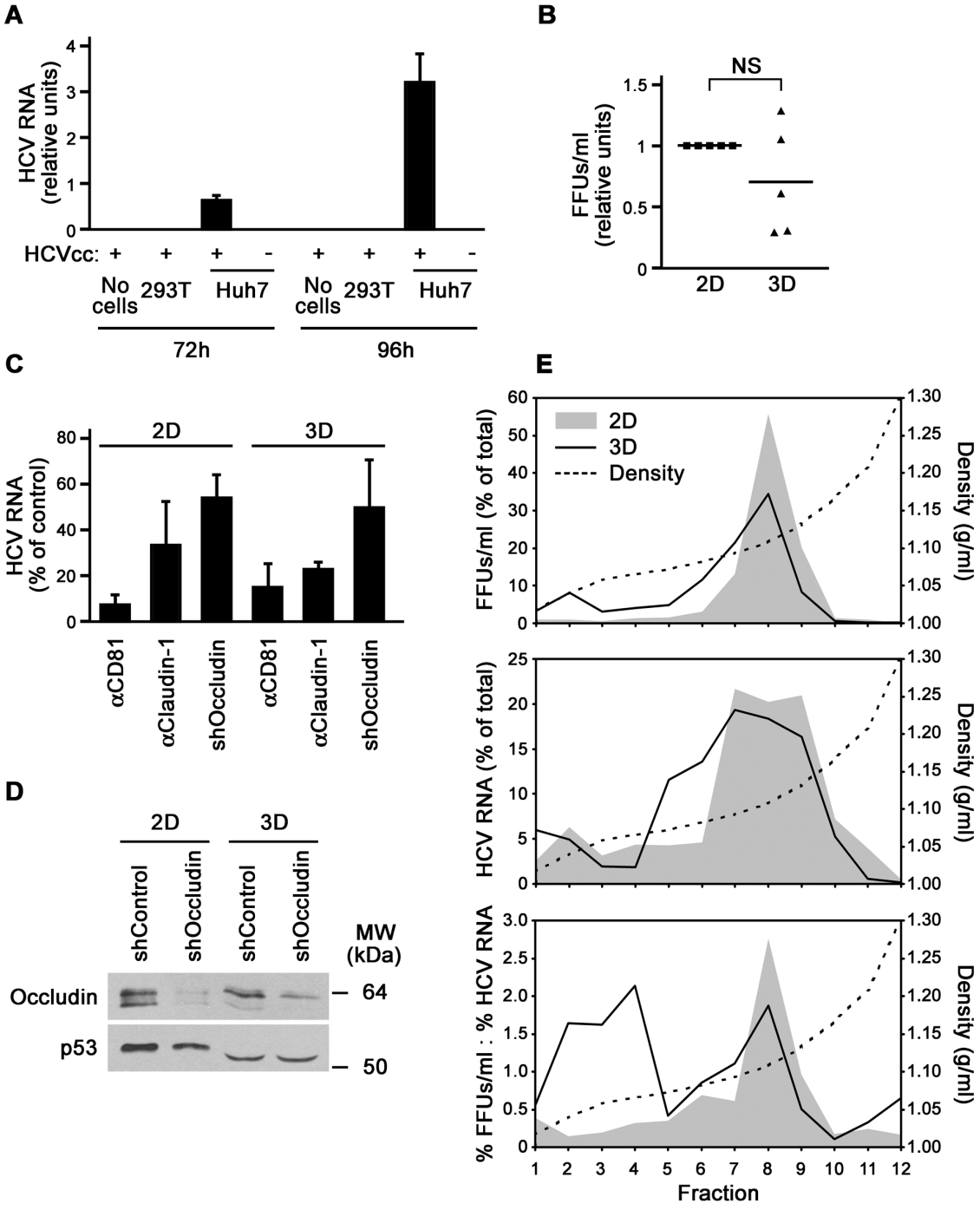


Fig. 4. Viral particle production by 3D cultures and comparison with 2D-generated HCVcc. (A) 3D cultures were performed with no cells and either 293T or Huh7 cells. After 6 days, cultures were infected overnight with 2D-generated HCVcc, washed and fresh complete medium was added. 72 and 96 h post-infection, culture supernatants were harvested, filtered and used to infect naïve 2D-cultured Huh7 cells. HCV RNA levels in target cells were determined 72 h later by real-time RT-PCR. Data are represented as the mean value ± SD of a triplicate experiment. **(B)** Huh7 cells were cultured in 2D or 3D conditions for 6 days and infected overnight with 2D-produced HCVcc. After washing, cells were cultured for 96 h and supernatants were harvested, filtered and titrated on 2D-cultured Huh7.5 cells. Data from five independent viral productions are expressed as relative units vs. 2D, and plotted as

black squares (2D) or triangles (3D) including the mean values (horizontal lines). NS, not statistically significant (*P*=0.84 vs. 2D, Mann-Whitney U test). **(C)** HCVcc was produced in 2D or 3D conditions as in (B) and used to infect naïve Huh7 cells that had been cultured in 2D or 3D conditions, respectively, for 6 days. Incubation of cultures with 2 µg/ml anti-CD81 or isotype control, or a 1/100 dilution of anti-claudin-1 or control serum, was performed 1 h prior 2D or 3D-produced HCVcc infection. Retroviral transduction of control and occludin shRNAs was performed twice, 32 and 8 h before target cell seeding. HCV RNA levels were measured by real-time RT-PCR 72 h after infection. Results are presented as the percentage of infection of either control antibody or control shRNA. Data are represented as the mean value ± SEM of at least two independent experiments performed in triplicate. **(D)** Occludin knockdown efficiency was evaluated by Western blot at the moment of 2D or 3D-produced HCVcc infection. **(E)** Viral supernatants from 2D (grey area) and 3D (solid line) cultures were subjected to ultracentrifugation through 0-40% iodixanol gradients. Fractions were used to measure infectivity (top panel) and HCV RNA levels (middle panel). FFUs, focus forming units. Values for each fraction are expressed as the percentage of the total of the gradient. Density is plotted as a dotted line in all panels. Data are represented as the mean value of at least four independent experiments. The bottom panel represents the ratio between infectivity (top panel) and HCV RNA levels (middle panel).

PLC/PRF/5 spheroids were infective, and that those collected 96 h after infection were more infective than when harvested 72 h post-infection (Fig. 4A and data not shown). Of note, supernatants derived from HCVcc-challenged Matrigel without cells or 3D-cultured 293T cells, which are not susceptible to HCVcc infection [20], were not infective. These data ruled out the possibility that supernatant infectivity was due to carry-over HCVcc from the inoculum. Additionally, side-by-side experiments with 2D and 3D cultures showed that HCVcc-infected 3D-cultured cells were able to generate infectious supernatants with similar titers (~10^3 FFUs/ml) as standard 2D cultures (Fig. 4B). Next, we wanted to determine whether molecules known to mediate HCV entry in standard 2D conditions are also necessary in a 3D context. As shown in Fig. 4C, anti-claudin-1 serum and anti-CD81 antibody inhibited HCVcc infection in both 2D and 3D cultures to a comparable extent. Similarly, occludin knockdown (Fig. 4D) markedly reduced the susceptibility of 2D and 3D-cultured Huh7 cells to HCVcc infection. These data strongly suggested that CD81, claudin-1 and occludin participated in HCVcc infection of hepatocyte-like polarized cultures.

Finally, in order to determine the density profile of 2D and 3D-generated HCVcc, culture supernatants were subjected to ultracentrifugation through continuous iodixanol gradients. The infectivity and HCV RNA content of each fraction was evaluated by titration on Huh7.5 cells and real-time RT-PCR, respectively. Whereas infectivity of

both 2D and 3D-produced HCVcc peaked at 1.107 g/ml, fractions below 1.107 g/ml obtained in 3D conditions consistently presented a higher associated infectivity than those derived from 2D cultures (Fig. 4E, top). Interestingly, 3D culture-derived supernatants showed an almost 6-fold increase in the relative infectivity of fractions between 1.017 and 1.058 g/ml compared to 2D conditions (Table 1). Additionally, HCV RNA peaked between 1.094 and 1.132 g/ml in both conditions, but 3D-obtained fractions between 1.072 and 1.082 g/ml contained approximately 3-fold higher viral RNA levels than their 2D counterparts (Fig. 4E, middle). When a ratio between infectivity and HCV RNA was calculated, values obtained from fractions ≥1.072 g/ml were similar in both conditions (Fig. 4E, bottom); however, 3D-derived fractions between 1.04 and 1.066 g/ml presented approximately 7- to 12-fold higher values than 2D-obtained fractions (Fig. 4E, bottom). Collectively, these data suggested that 3D-generated HCVcc shifted towards lower densities, and that specific infectivity between 1.04 and 1.066 g/ml was markedly increased in these conditions.

DISCUSSION

The ability to modulate hepatocyte polarity and multicellular organization is important for developing *in vitro* systems designed to reproduce liver functions [21]. Extracellular matrix modulates various cellular processes including polarization and cell metabolism, and has been shown to maintain the phenotype of hepatocytes in culture [22]. Herein, we demonstrate that Matrigel-embedded 3D cultures of Huh7 and PLC/PRF/5 cells, in contrast to standard 2D conditions, form polarized aggregates that develop TJ-delimited BC-like structures and are susceptible to HCVcc infection, constituting an improved system for the study of HCV and the virus-host interaction. In addition, it emerges as a suitable model to further characterize the contribution of polarity and TJ-associated proteins to HCV infection in a hepatocytic polarization context. Matrigel-embedded 3D aggregates are also able to produce infectious HCVcc particles. Moreover, infection in a 3D-context (i. e., 3D-generated virions employed to infect 3D cultures) is shown to be CD81, claudin-1 and occludin-dependent. These data strongly suggest that CD81, claudin-1 and occludin participate in HCVcc infection of cultures with hepatocytic polarization. These results are in agreement with recent studies in which serum-derived HCV infection of primary human hepatocytes (PHH) was claudin-1 and CD81 dependent [23, 24]. To our knowledge, this is the first demonstration that occludin is implicated in HCV infection of hepatocyte-like polarized cultures.

In plasma, HCV RNA-containing particles exhibit a wide range of density from 1.03 to 1.30 g/ml [4]. This heterogeneity has been attributed to a varying ratio of virions to non-enveloped cores and their variable association with host lipoproteins and/or immunoglobulins [4]. Interestingly, in the serum of an immunodeficient, anti-HCV negative patient the majority of viral RNA was found below 1.08 g/ml [25]. Additionally, a similarly narrow density range (<1.063 g/ml) was observed in a chimpanzee with a HCV acute infection prior to the onset of immune responses [26]. In contrast, viral RNA from HCVcc peaks at ~1.15 g/ml [16, 27-29]. Given that the higher density of *in vitro*-produced HCVcc could not be due to the presence of immunoglobulins complexed with the virions, these data suggest that HCVcc may differ from real HCV in terms of lipoprotein association and/or the percentage of total HCV RNA associated to naked capsids. Therefore, infectivity associated to high density HCVcc might correspond to viral particles that are significantly different from real virions, thus altering the readout of experiments. In this work, we have shown that both infectivity and viral RNA from 3D-generated HCVcc shift towards lower densities than HCVcc produced in 2D conditions. Similar results have been reported with viral particles obtained from either

PHH [30] or plasma from animals inoculated with standard HCVcc [29]. Moreover, we have shown that between 1.04 and 1.066 g/ml (fractions 2-4), despite similar relative levels of HCV RNA were found in both 2D and 3D conditions, the ratio infectivity/viral RNA was 7- to 12-fold higher for 3D-generated virions. This result suggests that infectivity of low density viral particles is higher when they derive from 3D cultures, observation which correlates well with the fact that particle density is inversely correlated to infectivity *in vivo* [26]. Collectively, these data indicate that the nature of host cells may determine some properties of the progeny virus, and that virions generated in a more physiological context better resemble natural HCV.

The very low density of some natural HCV particles has been attributed to an association of the virus with ApoB and/or ApoE-positive triglyceride-rich lipoproteins [4, 31-33] that might take place during viral egress [34]. Similar properties have also been reported with *in vitro* produced HCVcc in which virions were found to be secreted in a manner that parallels the formation of very low density lipoproteins (VLDLs) [28, 33-35]. On the other hand, early reports established a link among cell polarization, lipoprotein secretion and virus assembly. In highly polarized Caco-2 cells, lipoprotein secretion was shown to be a vectorial process mainly taking place across the basolateral membrane [36], and it could be enhanced by extracellular matrix proteins [37]. In addition, assembly of RNA enveloped viruses in MDCK cells was proved to be closely related to cell polarization [38]. Thus, it is reasonable to hypothesize that the lack of hepatocytic polarization of hepatoma-derived 2D-cultured cells could be responsible for a defective secretion of lipoproteins, which could in turn affect the correct assembly and egress of viral particles. In fact, it has been demonstrated that, in contrast to Huh7 and Huh7.5 cells, the polarized cell lines HepG2 and Caco-2 can secrete HCV glycoproteins in association with ApoB [39]. Furthermore, Huh7-derived cells have proved to be unable to secrete authentic, ApoB-containing VLDLs [30]. Interestingly, the different biophysical properties observed between 2D and 3D-generated virions were not related to major changes in differentiation-specific gene expression, but were most likely due to the modulation of the polarization state of virus-producing cells. This finding points to the existence of a direct link between HCV assembly and/or egress and cellular polarization regardless of the differentiation state. In summary, these observations suggest that *in vitro*-obtained viral particles from non-polarized cell lines may differ from both natural HCV and virions obtained from polarized cells in terms of lipoprotein composition or configuration within the lipoviroparticle, hypothesis which is currently being studied in our laboratory. This might explain why, in contrast to 2D-

produced HCV pseudotyped particles, HCV-like particles and HCVcc, infection with serum-derived HCV is an LDL receptor-dependent process [40-44].

It is largely assumed that PHH-derived systems are the best models to mimic natural HCV infection. However, working with PHH presents several limitations apart from ethical concerns and logistics requirements, including interindividual variability and differences in the quality of the available tissue that make it difficult to standardize both the isolation procedure and the obtained results [11]. Studying HCV life cycle in Huh7-derived 3D cultures presents the advantages of working with a cell line (homogeneity, unlimited availability, robust replication and virion production, easy genetic manipulation and the possibility of performing long term experiments) whilst maintaining hepatocytic polarity. Additionally, at least some characteristics of 3D-produced HCVcc are similar to those of natural HCV, thus providing a more physiologically relevant context than standard 2D cultures. Therefore, we propose Matrigel-embedded 3D Huh7 cultures as a practical system that could be used as a first screen before validating data with PHH. We expect that this novel, easy-made, high throughput-adaptable and reliable model will provide a valuable tool to understand the mechanisms governing hepatocyte HCV infection.

ACKNOWLEDGEMENTS

The authors express their gratitude to R. Bartenschlager and T. Wakita for providing us with critical reagents, and to A. Aguilera and J. Loureiro for statistical analysis.

REFERENCES

1. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. Lancet 2003;362:2095-2100.

2. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967-972.

3. Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature 2005;436:933-938.

4. Andre P, Perlemuter G, Budkowska A, Brechot C, Lotteau V. Hepatitis C virus particles and lipoprotein metabolism. Semin Liver Dis 2005;25:93-104.

5. Zeisel MB, Fofana I, Fafi-Kremer S, Baumert TF. Hepatitis C virus entry into hepatocytes: molecular mechanisms and targets for antiviral therapies. J Hepatol 2011;54:566-576.

6. Easter DW, Wade JB, Boyer JL. Structural integrity of hepatocyte tight junctions. J Cell Biol 1983;96:745-749.

7. Aijaz S, Balda MS, Matter K. Tight junctions: molecular architecture and function. Int Rev Cytol 2006;248:261-298.

8. Benedicto I, Molina-Jimenez F, Barreiro O, Maldonado-Rodriguez A, Prieto J, Moreno-Otero R, et al. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. Hepatology 2008;48:1044-1053.

9. Benedicto I, Molina-Jimenez F, Bartosch B, Cosset FL, Lavillette D, Prieto J, et al. The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection. J Virol 2009;83:8012-8020.

10. Perrault M, Pecheur EI. The hepatitis C virus and its hepatic environment: a toxic but finely tuned partnership. Biochem J 2009;423:303-314.

11. Gondeau C, Pichard-Garcia L, Maurel P. Cellular models for the screening and development of anti-hepatitis C virus agents. Pharmacol Ther 2009;124:1-22.

12. Sainz B, Jr., TenCate V, Uprichard SL. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. Virol J 2009;6:103.

13. Aizaki H, Nagamori S, Matsuda M, Kawakami H, Hashimoto O, Ishiko H, et al. Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. Virology 2003;314:16-25.

14. Murakami K, Ishii K, Ishihara Y, Yoshizaki S, Tanaka K, Gotoh Y, et al. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 2006;351:381-392.

15. Mee CJ, Harris HJ, Farquhar MJ, Wilson G, Reynolds G, Davis C, et al. Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells. J Virol 2009;83:6211-6221.

16. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.

17. Kleinman HK, Martin GR. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol 2005;15:378-386.

18. Wang W, Soroka CJ, Mennone A, Rahner C, Harry K, Pypaert M, et al. Radixin is required to maintain apical canalicular membrane structure and function in rat hepatocytes. Gastroenterology 2006;131:878-884.

19. Harris HJ, Davis C, Mullins JG, Hu K, Goodall M, Farquhar MJ, et al. Claudin association with CD81 defines hepatitis C virus entry. J Biol Chem;285:21092-21102.

20. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801-805.

21. Decaens C, Durand M, Grosse B, Cassio D. Which in vitro models could be best used to study hepatocyte polarity? Biol Cell 2008;100:387-398.

22. Castell JV, Gomez-Lechon MJ. Liver cell culture techniques. Methods Mol Biol 2009;481:35-46.

23. Fofana I, Krieger SE, Grunert F, Glauben S, Xiao F, Fafi-Kremer S, et al. Monoclonal anti-claudin 1 antibodies prevent hepatitis C virus infection of primary human hepatocytes. Gastroenterology;139:953-964, 964 e951-954.

24. Molina S, Castet V, Pichard-Garcia L, Wychowski C, Meurs E, Pascussi JM, et al. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent. J Virol 2008;82:569-574.

25. Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechockchai W, Toms GL. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. J Virol 2006;80:2418-2428.

26. Hijikata M, Shimizu YK, Kato H, Iwamoto A, Shih JW, Alter HJ, et al. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J Virol 1993;67:1953-1958.

27. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.

28. Gastaminza P, Kapadia SB, Chisari FV. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. J Virol 2006;80:11074-11081.

29. Lindenbach BD, Meuleman P, Ploss A, Vanwolleghem T, Syder AJ, McKeating JA, et al. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc Natl Acad Sci U S A 2006;103:3805-3809.

30. Podevin P, Carpentier A, Pene V, Aoudjehane L, Carriere M, Zaidi S, et al. Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. Gastroenterology.

31. Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76:6919-6928.

32. Diaz O, Delers F, Maynard M, Demignot S, Zoulim F, Chambaz J, et al. Preferential association of Hepatitis C virus with apolipoprotein B48-containing lipoproteins. J Gen Virol 2006;87:2983-2991.

33. Chang KS, Jiang J, Cai Z, Luo G. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. J Virol 2007;81:13783-13793.

34. Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W, Chisari FV. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J Virol 2008;82:2120-2129.

35. Meunier JC, Russell RS, Engle RE, Faulk KN, Purcell RH, Emerson SU. Apolipoprotein c1 association with hepatitis C virus. J Virol 2008;82:9647-9656.

36. Traber MG, Kayden HJ, Rindler MJ. Polarized secretion of newly synthesized lipoproteins by the Caco-2 human intestinal cell line. J Lipid Res 1987;28:1350-1363.

37. Ratcliffe DR, Iqbal J, Hussain MM, Cramer EB. Fibrillar collagen type I stimulation of apolipoprotein B secretion in Caco-2 cells is mediated by beta1 integrin. Biochim Biophys Acta 2009;1791:1144-1154.

38. Rodriguez-Boulan E, Paskiet KT, Sabatini DD. Assembly of enveloped viruses in Madin-Darby canine kidney cells: polarized budding from single attached cells and from clusters of cells in suspension. J Cell Biol 1983;96:866-874.

39. Icard V, Diaz O, Scholtes C, Perrin-Cocon L, Ramiere C, Bartenschlager R, et al. Secretion of hepatitis C virus envelope glycoproteins depends on assembly of apolipoprotein B positive lipoproteins. PLoS One 2009;4:e4233.

40. Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D, et al. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J Hepatol 2007;46:411-419.

41. Triyatni M, Saunier B, Maruvada P, Davis AR, Ulianich L, Heller T, et al. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. J Virol 2002;76:9335-9344.

42. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 2003;197:633-642.

43. Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, et al. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003;100:7271-7276.

44. von Hahn T, Lindenbach BD, Boullier A, Quehenberger O, Paulson M, Rice CM, et al. Oxidized low-density lipoprotein inhibits hepatitis C virus cell entry in human hepatoma cells. Hepatology 2006;43:932-942.

SUPPLEMENTARY INFORMATION

Supplementary Materials and Methods

Cell culture, generation of HCV replicon-containing clones and HCVcc infection

293T cells (ATCC CRL-11268) and human hepatocyte-derived cell lines Huh7 (JCRB 0403) and PLC/PRF/5 (ATCC CRL-8024) were grown at 37ºC with a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum, 2mM L-glutamine, 50 µg/ml gentamycin, 100 U/ml penicillin and 100 µg/ml streptomycin. For 3D Matrigel-embedded cultures, 50 µl of complete medium containing 5×10^3 cells was added to 50 µl of Growth factor-reduced Matrigel with a protein concentration ranging from 7 to 7.5 µg/µl (BD Biosciences, San Jose, CA), gently mixed and deposited into either a 8-well chambered coverglass (Nalge Nunc International, Rochester, NY) or a 48-well plate (Corning Incorporated, Corning, NY) depending on the experiment and incubated at 37°C for 30 minutes. For some experiments, 3D cultures were scaled to 24-well plates using 100 µl of complete medium containing 10^4 cells and 100 µl of Matrigel (see below). Cells were then covered with complete medium and grown for the indicated times, changing medium every 2 days. Huh7 cells expressing full-length or subgenomic genotype 1b (Con1, EMBL database accession number AJ238799) HCV replicons were established as previously described [8]. For HCVcc infection assays, 5×10^3 cells were grown on 2D (24-well plates) or 3D (48-well plates) cultures for 6 days. Where indicated, anti-CD81 antibody (clone JS-81, BD Biosciences), MOPC-21 mouse isotype control (Sigma, St. Louis, MO), polyclonal anti-claudin-1 or preimmune sera [45] were added 1 h before infection. Cultures were infected with 10^3 FFUs overnight, medium was changed and cells and supernatants were processed at different times as indicated.

Immunofluorescence analysis and confocal microscopy

Cells were grown in chambered coverglasses with or without Matrigel for 6 or 9 days. Cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature and permeabilized with 0.5% TritonX-100 in PBS for 10 minutes at room temperature. Cells were rinsed three times with 100 mM glycine in PBS for 15 minutes and blocked with TNB [0.1 M Tris-HCl, 0.15 M NaCl, 0.5% blocking reagent (Boehringer Mannheim GmbH, Mannheim,Germany)] plus 10% goat serum from Sigma for 2 h at 37ºC. Cells were incubated with the indicated antibodies diluted in TNB overnight at room

temperature and after washing with 0.1% NP-40 in PBS, cells were incubated with Alexa 488 or rhodamine X-conjugated goat anti-mouse or anti-rabbit antibodies (Molecular Probes, Inc., Eugene, OR) for 2 h at 37ºC and counterstained with DAPI (Pierce, Rockford, IL). For double stainings, samples were incubated sequentially with the indicated primary and secondary antibodies. The preparations were analyzed with a Leica TCS-SP (Leica Microsystems, Heidelberg, Germany) confocal microscope. Antibodies used were: monoclonal anti-Na^+, K^+-ATPase (Abcam, Cambridge, UK), anti-GM130 (BD Bioscencies), anti-MRP2 (M2 III-6; Alexis Laboratories, San Diego, CA), anti-CD81 (clone 1.3.3.22, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-occludin (Zymed, San Francisco, CA), polyclonal anti-radixin (Cell Signaling Technology, Beverly, MA), anti-ZO-1, anti-occludin, anti-claudin-1 (Zymed), anti-SR-BI (Novus Biologicals, Littleton, CO) and monoclonal antibody against HCV core (clone C7-50, Affinity BioReagents, Goleen, CO).

Western blots

5×10^3 cells were grown on 48-well plates for 3D Matrigel cultures or 24-well plates for 2D conventional cultures for 6 to 9 days. Cells were lysed on the plate with 100 µl of Laemmli buffer and boiled for 5 minutes. Western blots were carried out as previously described [8] with the following antibodies: polyclonal anti-occludin, anti-claudin-1 and anti-SR-BI and monoclonal anti-MRP2 (see above), anti-CD81 (clone 5A6) and anti-p53 (Santa Cruz Biotechnology).

Characterization of hepatocyte-like polarity

Functional BC, those that exhibited an accumulation of 5-chloromethylfluorescein di-acetate (CMFDA) or cholylglycylamido fluorescein (CGamF), were determined by confocal analysis of fluorescent compounds [46-48]. The nonfluorescent lipophilic CMFDA (Molecular Probes) passively penetrates the plasma membrane. Inside the cells, cytosolic esterases cleave off its acetate residues, thereby releasing the fluorescent and membrane-impermeable product 5-chloromethylfluorescein (CMF), which can react (e.g., with glutathione) to form fluorescent conjugates. This methylfluorescein-glutathione complex is then actively secreted by MRP2 [48]. CGamF was synthesized by binding FITC to glycocholate at the carboxyl group following a previously described method [49]. Cells were grown into chambered coverglasses with or without Matrigel for 6 days and incubated with 5 µM of either CMFDA or CGamF in

culture medium for 20 minutes at 37ºC. Cells were rinsed four times with fresh medium and intracellular accumulation of fluorescent products was analyzed with a confocal microscope.

RT-PCR

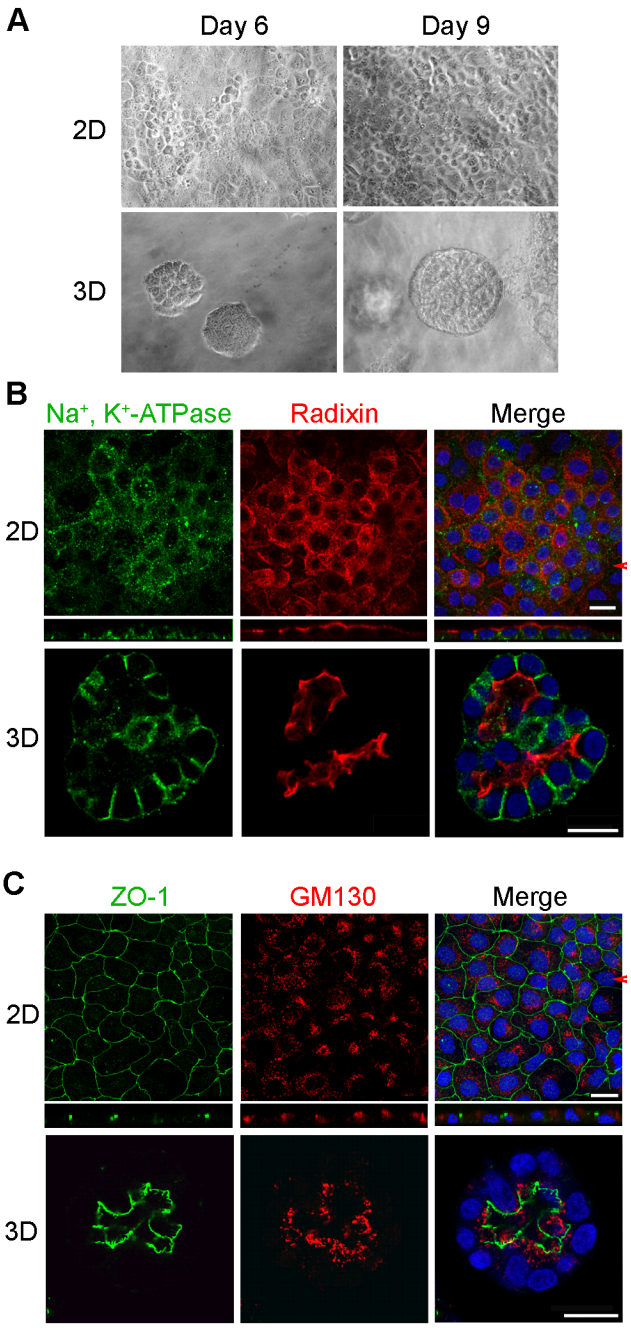
Specific primers used were: HCV, H3 [8] and MRP2 [50] as previously described; HNF4α, 5'-CGGAAGAACCACATGTACTC-3' and 5'-TGCTGTCCTCATAGCTTGAC-3'; α1AT, 5'-TCAAGGAGCTTGACAGAGAC-3' and 5'-GACAGCTTCTTACAGTGCTG-3'; TTR, 5'-GCTGGACTGGTATTTGTGTC-3' and 5'-ACTCACTGGTTTTCCCAGAG-3'; albumin, 5'-TGTGTTGCTGATGAGTCAGC-3' and 5'-ACATCAACCTCTGGTCTCAC-3'; CYP3A5, 5'-GGAGAGCACTAAGAAGTTCC-3' and 5'-TGTCGTTGAGGCGACTTTTC-3'; UGT1A1, 5'-ACAGAACTTTCTGTGCGACG-3' and 5'-CCACAATTCCATGTTCTCCAG-3'. Histone H3 mRNA levels were used for sample normalization.

Iodixanol gradient ultracentrifugation of 2D and 3D-generated HCVcc

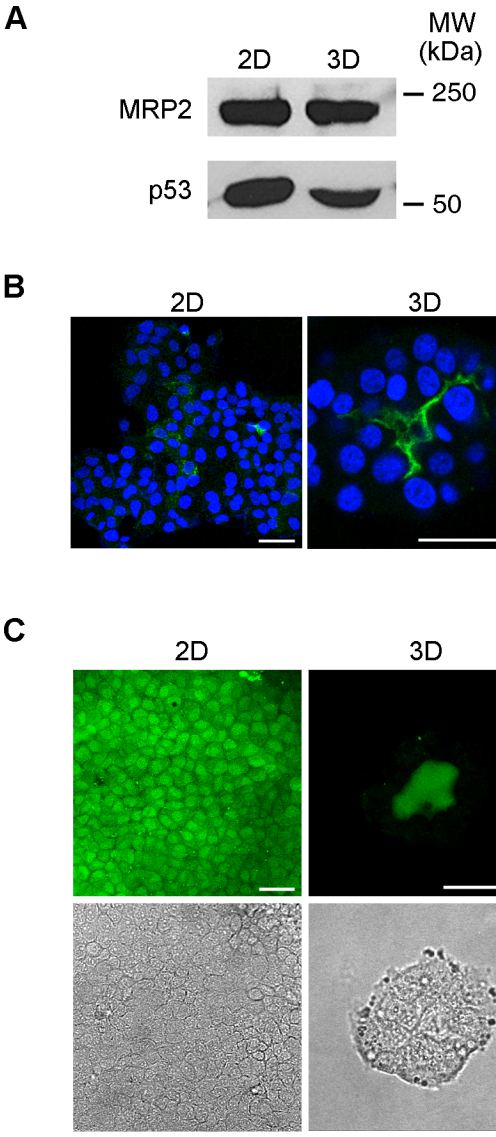
10^4 cells were seeded in 2D (12-well plates) and 3D (24-well plates) conditions. After 6 days, cells were HCVcc infected overnight, washed and 1 ml of complete medium per well was added. 96 h post-infection the supernatants were harvested, filtered through 0.45-µm pore-sized membranes and concentrated 5-fold in a 100,000 molecular weight cutoff Spin-X UF 6 Concentrator (Corning Incorporated) at 4ºC. Continuous 0-40% iodixanol gradients were prepared using Optiprep (Axis-Shield, Oslo, Norway). 5.5 ml of 40% iodixanol in 83 mM sucrose, 1 mM EDTA and 10mM Tris-HCl pH 7.4 were layered with 5.5 ml of homogenization media (250 mM sucrose, 1 mM EDTA and 10mM Tris-HCl pH 7.4) and the gradients were formed with a Gradient Master (BioComp Instruments, Inc., Fredericton, Canada). 1 ml of the concentrated viral supernatant was loaded on top of the gradient and tubes were subjected to ultracentrifugation in a SW41 rotor (Beckman Coulter, Fullerton, CA) at 31,000 rpm and 4ºC for 16 h. 1 ml fractions were collected from the top and titration was performed by infection of Huh7.5 cells with serially diluted fractions and staining with anti-core antibody. Viral RNA levels were measured by extracting RNA from 100 µl of each fraction with 900 µl of TRI Reagent (Ambion Inc., Austin, TX), using 20 µg of glycogen (Roche Diagnostics GmbH, Mannheim, Germany) as a carrier for RNA precipitation,

and performing real-time RT-PCR (see above). Density of fractions was calculated using a refractometer (EUROMEX microscopen BV, Arnhem, Holland).

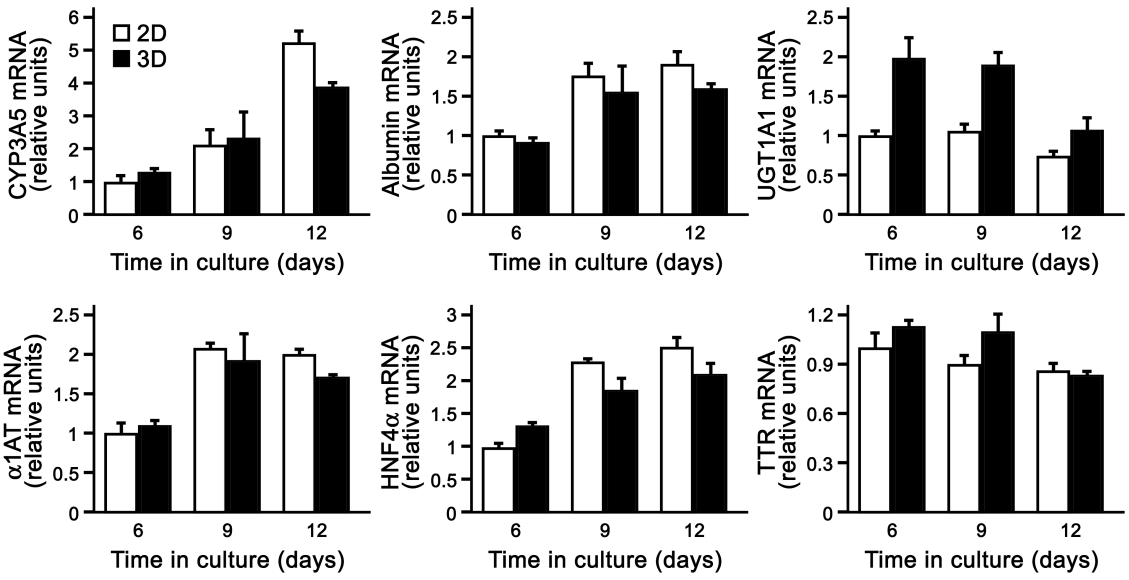
Supplementary Figures



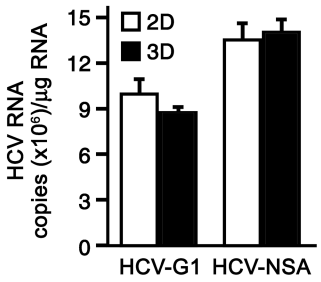
Supplementary Fig. S1. Characterization of 3D aggregates in the human hepatocyte-derived cell line PLC/PRF/5. (A) Phase contrast image (20x magnification) of 2D and 3D cultures 6 and 9 days after seeding. **(B and C)** Confocal immunofluorescence analysis of the distribution of membrane-associated proteins and cellular compartments. PLC/PRF/5 cells were grown in 2D and 3D conditions for 6 days and processed for double-label immunofluorescence. Na^+, K^+-ATPase and ZO-1, green; Radixin and GM130 (a Golgi matrix protein), red; nuclei were stained with DAPI (blue). 2D images show the merged projection of confocal stacks (top) and X-Z sections (bottom). Arrows indicate the plane from which the Z sections were taken. 3D images show a single X-Y section from the confocal stacks. Z sections were compiled by taking 0.5 μm steps through each X-Y section. Photographs are representative of at least three separate experiments. Bar, 25 μm.



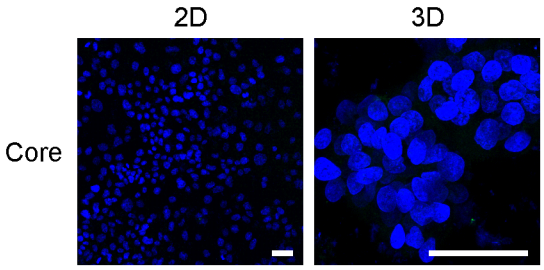
Supplementary Fig. S2. BC structure and function in 2D and 3D-cultured PLC/PRF/5 cells. (A) MRP2 protein levels were analyzed by Western blot. p53 was used as loading control. Molecular weight markers (kDa) are indicated on the right. **(B)** MRP2 localization in PLC/PRF/5 cells after 6 days of 2D and 3D culture was analyzed by confocal immunofluorescence analysis. Images show the merged projection of confocal stacks. MRP2, green; nuclei, blue. Bar, 50 μm. **(C)** PLC/PRF/5 cells were 2D or 3D cultured for 6 days, treated with 5 μM CMFDA and washed. Confocal images show the accumulation of CMFDA. Bar, 50 μm. Results shown are representative of at least three separate experiments.



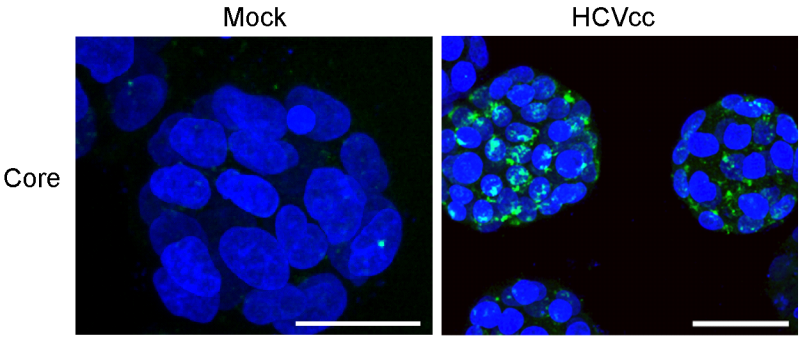
Supplementary Fig. S3. Analysis of the expression of hepatocyte-specific markers in 2D and 3D cultures. Huh7 cells were cultured in 2D or 3D conditions for 6, 9 or 12 days. RNA was extracted and real-time RT-PCR was performed. Data are represented as the mean value ± SD of two experiments.



Supplementary Fig. S4. HCV replication in 2D and 3D conditions. Genomic (HCV-G1) and subgenomic (HCV-NSA) HCV clones were 2D or 3D-cultured for 6 days. HCV RNA levels were measured by real-time RT-PCR. Data are represented as



Supplementary Fig. S5. Mock infection control. Huh7 cells were cultured in 2D or 3D conditions for 6 days and mock infected. Three days later, cells were processed for immunostaining using anti-core specific Ab (green). Nuclei were stained with DAPI (blue). Images show the merged projection of confocal stacks. Bar, 50 µm. Results are representative of two separate experiments.



Supplementary Fig. S6. PLC/PRF/5 3D cultures are susceptible to HCV infection. Cells were cultured in 3D conditions for 6 days and infected with HCVcc. A mock infection control was included. Three days after infection, cells were processed for immunostaining using anti-core specific Ab (green). Nuclei were stained with DAPI (blue). The image shows the merged projection of confocal stacks. Bar, 50 µm. Results are representative of two separate experiments.

Supplementary References

45. Krieger SE, Zeisel MB, Davis C, Thumann C, Harris HJ, Schnoberet EK, et al. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-Claudin-1 associations. Hepatology 2010; 51:1144-1157.

46. Bravo P, Bender V, Cassio D. Efficient in vitro vectorial transport of a fluorescent conjugated bile acid analogue by polarized hepatic hybrid WIF-B and WIF-B9 cells. Hepatology 1998;27:576-583.

47. Lindenmaier H, Becker M, Haefeli WE, Weiss J. Interaction of progestins with the human multidrug resistance-associated protein 2 (MRP2). Drug Metab Dispos 2005;33:1576-1579.

48. Bogman K, Erne-Brand F, Alsenz J, Drewe J. The role of surfactants in the reversal of active transport mediated by multidrug resistance proteins. J Pharm Sci 2003;92:1250-1261.

49. Sherman IA, Fisher MM. Hepatic transport of fluorescent molecules: in vivo studies using intravital TV microscopy. Hepatology 1986;6:444-449.

50. Briz O, Cassio D, Blazquez AG, Grosse B, Serrano MA, Marin JJ. Characterization of WIF-B9/R cells as an in vitro model with hepatocyte-like polarity and enhanced expression of canalicular ABC transporters involved in phase III of hepatic detoxification. Toxicology 2007;232:24-36.

DISCUSIÓN

DISCUSIÓN

El mantenimiento de la polaridad de los hepatocitos es crucial para la síntesis de la bilis y su secreción a los canalículos biliares. La separación eficaz entre los dominios apicales y basolaterales de los hepatocitos es fundamental en este proceso, ya que permite la correcta localización de los diferentes transportadores y la formación de los gradientes osmóticos necesarios para que se produzca una adecuada secreción biliar (206). Las TJs desempeñan un papel clave en la preservación de la polaridad de los hepatocitos y sellan los canalículos biliares impidiendo el contacto entre la sangre y la bilis. Tanto en muestras de pacientes como en diferentes modelos experimentales, se ha descrito que la alteración de la integridad estructural y funcional de las TJs está estrechamente relacionada con la aparición de colestasis, afección caracterizada por la disminución o interrupción del flujo de bilis (8, 178, 189). Por ello, el hecho de que HCV induzca la desorganización de las TJs y la acumulación intracelular de ocludina *in vitro* (25) (ver Anexo 1) podría explicar el desarrollo de hepatitis colestática que se produce en algunos casos durante la recidiva de HCV postransplante hepático donde la carga viral es particularmente elevada debido al proceso de inmnosupresión (54). En cualquier caso, esta atractiva hipótesis no ha podido ser confirmada en un estudio reciente llevado a cabo con biopsias de pacientes reinfectados por HCV tras transplante hepático (151). Sin embargo, tampoco se puede descartar su validez ya que es posible que el procesamiento a altas temperaturas de las muestras parafinadas afecte a las estructuras membranosas intracelulares e impida su correcta visualización. De hecho, dependiendo del protocolo de recuperación antigénica empleado durante la inmunohistoquímica, la localización subcelular aparente de claudina-1 en los hepatocitos varía considerablemente (151, 181). Por otra parte, cabe la posibilidad de que la deslocalización de proteínas asociadas a TJs mediada por HCV en el parénquima hepático sea menos pronunciada que la observada *in vitro* o que afecte a un menor número de células. De hecho, en pacientes con cargas virales $\geq 10^5$ UI/ml, el porcentaje de hepatocitos donde se detecta la presencia de proteínas virales se ha estimado entre 7 y 20% (125). Por ello, los posibles efectos de HCV sobre la integridad de las TJs en los hepatocitos *in vivo* pueden ser difíciles de detectar, lo que no excluye que existan y tengan importancia en la patogénesis de la infección por HCV. Aparte de la posible aparición de hepatitis colestática, la alteración de las TJs puede interferir con rutas de señalización implicadas en proliferación y diferenciación celular (142), lo que a su vez podría tener implicaciones en el desarrollo de hepatocarcinoma asociado a la infección por HCV. Además de perjudicar el

correcto funcionamiento del hígado, la perturbación de las TJs podría desempeñar algún papel en el ciclo viral, favoreciendo la propagación y supervivencia del virus. De forma semejante a los adenovirus y rotavirus (161, 217), es posible que HCV induzca la desarticulación de las TJs para facilitar la diseminación viral o el acceso del virus a sus receptores. Asimismo, la interacción intracelular de ocludina con la proteína E2 de la envuelta de HCV (25) podría tener un papel en la salida del virus de la célula infectada ya que, aprovechando el transporte de ocludina hacia la membrana plasmática, los viriones se podrían posicionar en zonas aptas para su exocitosis.

La asociación observada entre E2 y ocludina dio pie a estudiar su posible función como co-receptor de HCV. Esta hipótesis fue confirmada simultáneamente por tres grupos diferentes, incluido el nuestro (26, 130, 173) (ver Anexo 2). Además se describió que ocludina y CD81 influyen en el tropismo específico de especie (humanos y primates) de la infección por HCV (66, 154, 173), aunque posteriormente se demostró que tanto ocludina de tupaya como la versión canina pueden funcionar como co-receptores de HCV de forma similar a su ortólogo humano (154, 204). Por otra parte, se observó que el segundo dominio extracelular de ocludina es esencial para su función como co-receptor viral y su interacción con E2 (129, 154). Además se ha sugerido que ocludina, mediante su interacción con dinamina II, pueda promover la endocitosis de las partículas virales (129), aunque esta hipótesis no ha podido ser confirmada hasta el momento. Por último, se ha postulado que también la transmisión de HCV célula-célula es dependiente de ocludina, claudina-1, SR-BI y CD81 (33), aunque el papel de CD81 en este tipo de diseminación viral resulta controvertido (203, 220).

La identificación de ocludina como proteína necesaria para la infección por otros virus como el virus Coxsackie tipo B3 (CVB) (45) hizo pensar que quizás CVB y HCV podrían tener en común algún aspecto de sus mecanismos de entrada en la célula diana. Se observó que CVB, tras interaccionar con la proteína “decay-accelerating factor” (DAF) en la superficie apical de las células de epitelio intestinal Caco-2, el complejo se desplazaba lateralmente mediante la activación de las Rho GTPasas y la reorganización del citoesqueleto de actina para concentrarse en las TJs, desde donde se producía su internalización junto con ocludina de forma dependiente de CAR y caveolina-1. En ausencia de CAR, ocludina o caveolina-1, CVB permanecía retenido en las TJs (44, 45). En analogía con este mecanismo, se describió que una forma soluble de la proteína E2 de HCV se relocalizaba junto con CD81 a las áreas de contacto celular, y que tanto la activación de RhoA y Cdc42 como la remodelación del

citoesqueleto de actina eran necesarias para este proceso (32). No obstante, este desplazamiento hacia las uniones célula-célula no se pudo confirmar con partículas virales completas de HCV marcadas fluorescentemente, observándose que la entrada de éstas en la célula diana ocurría preferentemente en áreas excluidas de las uniones intercelulares (41). Sin embargo cabe destacar que la técnica utilizada en este último estudio no permite diferenciar la entrada viral productiva, que da lugar a la infección efectiva de las células, de la no productiva. Por ello, no se puede descartar la posibilidad de que un número reducido de virus penetren en la célula desde las zonas de contacto intercelular, y que estas partículas virales sean las responsables de la infección productiva. Por otra parte, las infecciones por CVB y HCV se asemejan en que, a pesar de que ambas dependen de ocludina, en ninguno de los dos casos se ha podido demostrar hasta la fecha su interacción directa con proteínas virales (45, 129). Además, la interferencia de su expresión no afecta a la unión del virus a la célula diana (26, 45). Es interesante el hecho de que ocludina participe en la infección por diferentes virus cuyo acceso a sus células diana es topológicamente distinto. En el caso de CVB, la partícula viral contacta con la célula del epitelio intestinal por su superficie luminal/apical, desde la que se desplaza hacia la zona de las TJs donde se internaliza junto con ocludina. Por el contrario, HCV presumiblemente establece contacto con los hepatocitos por su lado basolateral, en contacto con la sangre sinusoidal. Esto podría indicar que, independientemente de la ruta seguida, los virus se posicionan en las TJs y allí hacen uso de ocludina para penetrar en la célula. Esto podría sugerir que la TJ es una estructura involucrada en el proceso infectivo, lo que se ha demostrado para diferentes virus y bacterias (84, 90). El sorprendente carácter altamente dinámico de la estructura de las TJs (195), consideradas inicialmente como meros complejos estructurales, así como el continuo reciclaje endocítico de ocludina (157), podrían explicar su participación en la internalización de las partículas virales.

A diferencia de CVB, la infección por HCV depende también de claudina-1 (45, 59), y la entrada del virus en la célula ocurre a través de endocitosis mediada por clatrina (29, 150) con la participación de dinamina II (129). Por todo ello, aunque CVB y HCV hagan uso de ocludina para penetrar en la célula diana, no parece que el mecanismo de entrada sea el mismo. De hecho, a pesar de que la infección por HCV requiere la presencia de las proteínas asociadas a TJs claudina-1 y ocludina, el papel de las TJs en sí durante la entrada viral resulta controvertido. Aunque existen trabajos donde se sugiere que la integridad de las TJs es necesaria para la infección por HCV (32, 46, 194), estudios realizados en células polarizadas ponen en duda esta hipótesis (146-148). Por una parte, se ha observado que la interacción de claudina-1 con CD81 es

necesaria para que se produzca la asociación estable del virión con la superficie celular y la consiguiente entrada viral (92, 116), y que la unión de CD81 y claudina-1 ocurre predominantemente en zonas de la membrana plasmática fuera de las TJs (92). Sin embargo, este último dato no ha podido ser confirmado en PHHs (93). De hecho existe controversia en cuanto a la localización de claudina-1 en los hepatocitos, ya que mientras que algunos estudios con biopsias han mostrado su presencia tanto en las TJs como en la membrana basolateral de los hepatocitos (93, 181), en otros trabajos se ha detectado exclusivamente en las TJs (71, 151). Por otro lado, se ha descrito que el factor de crecimiento del endotelio vascular (VEGF) induce la despolarización celular y favorece la infección por HCV (146). Sin embargo, también se ha podido observar que el tratamiento con VEGF promueve la localización intracelular de ocludina (146, 159), posiblemente como resultado de su endocitosis desde las TJs. Por ello, estos datos no permiten discriminar si el incremento de la infección inducido por VEGF pudiera ser debido a la pérdida de polarización celular en sí o a la inducción de la internalización de ocludina, lo que podría mediar la endocitosis viral. No obstante, los datos obtenidos hasta el momento no parecen apoyar esta última hipótesis, ya que el dominio C-terminal de ocludina necesario para su internalización dependiente de VEGF (159) no es indispensable para la entrada de HCV en la célula diana (110, 129). Sin embargo, cabe destacar que estos estudios se realizaron mediante expresión ectópica de mutantes de ocludina en células 786-O, una línea renal que no expresa ocludina de forma endógena y no posee TJs ni presenta un fenotipo polarizado. Por ello, es posible que las células 786-O y los hepatocitos polarizados difieran considerablemente en cuanto a localización, endocitosis y/o reciclaje de ocludina, por lo que las conclusiones obtenidas en estos trabajos deben ser interpretadas cuidadosamente.

El mecanismo a través del cual ocludina participa en la infección por HCV sigue siendo una incógnita, y hasta el momento su análisis ha estado dificultado por la ausencia de herramientas como un anticuerpo frente a ocludina que bloquee la entrada viral. Además de la posible internalización conjunta de la partícula viral y ocludina expuesta anteriormente, cabría imaginar otras hipótesis alternativas que explicaran la participación de ocludina en la infección por HCV. El hecho de no haberse podido detectar una asociación directa entre ocludina y las proteínas de la envuelta de HCV (129) podría sugerir que, de manera semejante al complejo partícula viral-CD81-claudina-1, la interacción de ocludina con otro co-receptor viral (X) fuera necesaria para la formación de un complejo partícula viral-X-ocludina que mediara la entrada de HCV en la célula. Una posibilidad consistiría en que ocludina dirigiera dicho complejo

hacia una zona de la membrana plasmática donde tendría lugar la endocitosis del virión. Esta zona podría estar definida bien por la presencia de un tercer co-receptor necesario para la internalización viral, o bien por constituir un dominio dinámico con un elevado número de procesos endocíticos, como la TJ (195). De hecho, este tipo de transporte lateral previo a la entrada de la partícula viral en la célula ha sido descrito para numerosos virus (34). Otra posibilidad sería que la inclusión de ocludina en el complejo macromolecular de entrada viral propiciara un cambio conformacional en algún factor de la célula huésped o en las proteínas de la envuelta de HCV, de manera que se posibilitara la endocitosis del virión y/o su fusión con la membrana endosomal. Es interesante destacar que mediante ensayos de fusión célula-célula se ha observado que ocludina participa en la fusión de membranas dependiente de las proteínas de envuelta de HCV (26). Curiosamente, este fenómeno también se ha descrito para CD81 y claudina-1 (59, 109), lo que podría indicar la existencia de un conjunto coordinado de proteínas que favoreciera el proceso de fusión durante la entrada viral. En ese sentido, se sabe que la proteína gp120 de la envuelta del virus de la inmunodeficiencia humana (HIV) sufre un cambio conformacional tras interaccionar con CD4, presente en la membrana de la célula diana, lo que posibilita su posterior asociación con los co-receptores CXCR4 o CCR5 y la fusión de las membranas virales y celulares (57).

Con el objetivo de diseñar estrategias antivirales eficientes es necesario adquirir un conocimiento detallado de los mecanismos de la infección por HCV, siendo especialmente importante descifrar el modo en el que los factores de la célula huésped participan en dicho proceso. La intervención terapéutica sobre éstos podría eludir el problema que supone la gran variabilidad genética del virus, que impide una respuesta inmune eficaz y posibilita la adaptación viral a los tratamientos mediante el surgimiento de cepas resistentes (225). En los últimos años, el desarrollo de herramientas para estudiar el ciclo de HCV *in vitro* ha permitido un avance muy significativo en la identificación de factores celulares implicados en diferentes fases de la infección. No obstante, la interpretación de los resultados debe ser realizada con cautela ya que los datos obtenidos difieren considerablemente dependiendo del sistema experimental empleado para el estudio. Por ejemplo, aunque se ha descrito que la infectividad de HCVpp depende de la presencia de un citoesqueleto de actina intacto (41), este requerimiento no ha sido observado para la infección con HCVcc (185). Por otra parte, el grado de participación de LDL-R en el proceso infectivo es muy variable en función del sistema experimental empleado, lo que incluye tanto la naturaleza de las partículas infectivas (HCVser, HCVcc o HCVpp) como las células diana (líneas celulares o PHHs)

(2, 22, 99, 155, 214). El sistema de HCVpp, a pesar de ser muy útil para estudiar la entrada viral, presenta algunas limitaciones. Los mecanismos de ensamblaje de los retrovirus y lentivirus empleados para generar las HCVpp son muy diferentes de las rutas seguidas en la biogénesis de HCV, lo que posiblemente influya en la composición de ambas partículas virales y tenga consecuencias funcionales en su interacción con la célula diana. En cuanto a las discrepancias observadas entre HCVser y HCVcc, éstas podrían tener su origen en el hecho de que los hepatocitos, las células en las que tiene lugar el ciclo de HCV, presentan unas características muy especiales que no se conservan en la mayoría de sistemas *in vitro*. Así, las células Huh7 y sus derivados, las más empleadas como fuente de HCVcc, no presentan la polarización típica de los hepatocitos (25, 50) ni tampoco son capaces de secretar auténticas VLDLs (175). Dada la relación que se ha establecido entre polarización y secreción de lipoproteínas (180, 205) y la interconexión descrita entre la biogénesis de VLDLs y el ensamblaje de HCV (47, 76, 77, 100, 153), es razonable pensar que una polarización deficiente de las células productoras puede conllevar la generación de partículas virales defectivas, con una composición diferente al HCV proveniente de hepatocitos polarizados. De hecho, se ha demostrado que, a diferencia de las células polarizadas Caco-2 y HepG2, la línea Huh7 no es capaz de secretar las proteínas de envuelta de HCV acomplejadas con ApoB (101). Por ello, la diferente composición y/o disposición de las lipoproteínas presentes en HCVcc generado en células Huh7 y en HCVser podría justificar los distintos resultados obtenidos con ambos sistemas, especialmente los concernientes a la dependencia de LDL-R para su infectividad.

Además de la posible alteración de la partícula infectiva en función del grado de polarización de las células productoras, es necesario tener en cuenta que los mecanismos de infección también pueden estar relacionados con la polarización de las células diana. En el caso de HCV, esta consideración adquiere una relevancia especial ya que claudina-1 y ocludina, proteínas asociadas a TJs, actúan como co-receptores virales. A pesar de que actualmente existe controversia con respecto al posible papel de la polaridad celular en la entrada viral, parece lógico pensar que la polarización de las células productoras de HCV, la síntesis y secreción de lipoproteínas, la composición del virión y sus mecanismos de entrada en el hepatocito polarizado pueden estar íntimamente relacionados (ver Anexo 3). Por ello, para obtener conclusiones fiables, es preciso estudiar el ciclo completo de HCV en un contexto donde estos factores se asemejen lo máximo posible a los hepatocitos y a las partículas de HCV presentes en el suero de los pacientes. Respecto a los sistemas *in vivo*, el restringido tropismo de especie de HCV ha dificultado el desarrollo de modelos

murinos de infección. Recientemente se ha generado un modelo en ratón con el hígado y el sistema inmune humanizados, que es susceptible de infectarse por HCVser, generar una respuesta inmune específica contra el virus y desarrollar hepatitis y fibrosis hepática (218). A pesar de que este y otros modelos *in vivo* constituyen una valiosa herramienta para el estudio de la infección por HCV, su complejidad técnica y el hecho de necesitar el aporte de células primarias humanas (progenitores hepáticos y células madre hematopoyéticas) lo convierte en un sistema apto para validar datos pero poco conveniente para el estudio rutinario de HCV y la investigación de los mecanismos moleculares implicados en el ciclo viral.

In vitro, el uso de PHHs se considera muy adecuado para el estudio de la infección por HCV ya que las células se pueden mantener en un estado de alta polarización y diferenciación y son susceptibles de ser infectadas por HCVser, HCVcc y HCVpp (82). Además, recientemente se ha descrito que es posible generar nuevas partículas virales infectivas a partir PHHs infectados (175), por lo que este sistema es válido para estudiar el ciclo viral completo. Sin embargo, las limitaciones intrínsecas de las células primarias humanas, como su restringida disponibilidad o la elevada heterogeneidad entre muestras, dificultan el uso de PHHs como sistema cotidiano para el estudio de HCV. Por ello, son necesarios sistemas experimentales *in vitro* que estén basados en el uso de líneas celulares pero que a su vez sus características se asemejen lo máximo posible a las de los hepatocitos. La línea celular HepG2 con expresión ectópica de CD81 ha sido utilizada como modelo para el estudio de la infección por HCV en un contexto de polaridad hepatocitaria (146, 148), aunque su limitada susceptibilidad a la infección (unas 700 veces menor que la de las células Huh7.5) (127, 148) imposibilita su uso para el estudio del ensamblaje y secreción del virus. Por otro lado, se ha conseguido reproducir el ciclo viral completo en cultivos tridimensionales (3D) realizados en biorreactores (5, 158, 187). Estos trabajos, a pesar de mostrar un incremento en la expresión de genes específicos de diferenciación en los cultivos 3D comparados con los cultivos tradicionales bidimensionales, no han demostrado que aquéllos presenten un estado de polarización semejante al de los hepatocitos.

El uso de Matrigel para el cultivo de hepatocitos primarios y líneas celulares derivadas de hepatocitos ha sido empleado extensivamente con la finalidad de mantener la polaridad, diferenciación y funcionalidad de las células *in vitro* (108), aunque su utilidad para el estudio de la infección por HCV no había sido evaluada hasta el momento. Nosotros hemos demostrado que los cultivos 3D de líneas celulares incluidas en

Matrigel, además de adquirir la polarización estructural y funcional típica de los hepatocitos, eran susceptibles de ser infectados por HCVcc y producir nuevas partículas virales infectivas con títulos similares a los obtenidos en cultivos bidimensionales estándar (ver Anexo 4). Por una parte, estos cultivos permiten analizar los requerimientos celulares necesarios para que la infección tenga lugar en un contexto de polaridad hepatocitaria. Por otro lado, posibilitan el estudio de los efectos del virus sobre la célula huésped en unas condiciones más parecidas a las del hepatocito en su entorno natural. Además, este sistema puede ser empleado para caracterizar en detalle las partículas virales generadas a partir de células polarizadas. Esta última utilidad puede ser de gran interés, ya que se ha observado que, en comparación con las partículas virales provenientes de cultivos tradicionales bidimensionales, en los virus generados a partir de líneas celulares cultivadas en 3D se observa la aparición de partículas virales con una menor densidad y una mayor infectividad específica (ver Anexo 4). Cabe destacar que estas características son semejantes a las de los virus producidos por PHHs inoculados con HCVcc (175) y los presentes en el suero de pacientes (9, 30, 97, 162) o de animales infectados (128). Por ello, estos datos sugieren que tanto las propiedades estructurales como funcionales de los viriones pueden depender de las características de las células productoras. Así, los mecanismos y requerimientos moleculares de la infección podrían ser diferentes según la procedencia de las partículas virales, lo que tendría especial importancia a la hora de elegir sistemas experimentales adecuados para evaluar la eficacia de compuestos antivirales y diseñar nuevas estrategias contra HCV.

CONCLUSIONES

CONCLUSIONES

1) Las proteínas estructurales de HCV, posiblemente las de la envuelta viral, inducen alteraciones reversibles en la línea celular derivada de hepatocitos humanos Huh7 tanto en la localización subcelular de las proteínas asociadas a TJs ocludina, claudina-1 y ZO-1 como en las funciones características de las TJs.

2) Las proteínas estructurales de HCV inducen la acumulación de ocludina en el retículo endoplásmico de células Huh7, observándose una interacción entre ocludina y la proteína de la envuelta viral E2.

3) Ocludina actúa como co-receptor de HCV participando en la entrada del virus en la célula huésped, probablemente durante una fase tardía de la misma.

4) El cultivo tridimensional de líneas celulares derivadas de hepatocitos humanos incluidas en Matrigel constituye un sistema para el estudio de HCV donde las células presentan la polarización típica de los hepatocitos y el ciclo viral tiene lugar de forma completa.

5) La infección por HCV en un contexto tridimensional depende de la presencia de CD81, claudina-1 y ocludina en la célula diana.

6) Las partículas de HCV generadas a partir de cultivos tridimensionales, en comparación con el virus obtenido en condiciones de cultivo estándar, son más parecidas a las existentes en pacientes en términos de densidad e infectividad específica.

BIBLIOGRAFÍA

BIBLIOGRAFÍA

1. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 271:518-20.

2. Agnello, V., G. Abel, M. Elfahal, G. B. Knight, and Q. X. Zhang. 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci U S A 96:12766-71.

3. Aijaz, S., M. S. Balda, and K. Matter. 2006. Tight junctions: molecular architecture and function. Int Rev Cytol 248:261-98.

4. Aizaki, H., K. Morikawa, M. Fukasawa, H. Hara, Y. Inoue, H. Tani, K. Saito, M. Nishijima, K. Hanada, Y. Matsuura, M. M. Lai, T. Miyamura, T. Wakita, and T. Suzuki. 2008. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 82:5715-24.

5. Aizaki, H., S. Nagamori, M. Matsuda, H. Kawakami, O. Hashimoto, H. Ishiko, M. Kawada, T. Matsuura, S. Hasumura, Y. Matsuura, T. Suzuki, and T. Miyamura. 2003. Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. Virology 314:16-25.

6. Akazawa, D., T. Date, K. Morikawa, A. Murayama, M. Miyamoto, M. Kaga, H. Barth, T. F. Baumert, J. Dubuisson, and T. Wakita. 2007. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 81:5036-45.

7. Amako, Y., K. Tsukiyama-Kohara, A. Katsume, Y. Hirata, S. Sekiguchi, Y. Tobita, Y. Hayashi, T. Hishima, N. Funata, H. Yonekawa, and M. Kohara. 2010. Pathogenesis of hepatitis C virus infection in Tupaia belangeri. J Virol 84:303-11.

8. Anderson, J. M. 1996. Leaky junctions and cholestasis: a tight correlation. Gastroenterology 110:1662-5.

9. Andre, P., F. Komurian-Pradel, S. Deforges, M. Perret, J. L. Berland, M. Sodoyer, S. Pol, C. Brechot, G. Paranhos-Baccala, and V. Lotteau. 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 76:6919-28.

10. Andre, P., G. Perlemuter, A. Budkowska, C. Brechot, and V. Lotteau. 2005. Hepatitis C virus particles and lipoprotein metabolism. Semin Liver Dis 25:93-104.

11. Andreo, U., P. Maillard, O. Kalinina, M. Walic, E. Meurs, M. Martinot, P. Marcellin, and A. Budkowska. 2007. Lipoprotein lipase mediates hepatitis C virus (HCV) cell entry and inhibits HCV infection. Cell Microbiol 9:2445-56.

12. Appel, N., T. Schaller, F. Penin, and R. Bartenschlager. 2006. From structure to function: new insights into hepatitis C virus RNA replication. J Biol Chem 281:9833-6.

13. Balda, M. S., and K. Matter. 2008. Tight junctions at a glance. J Cell Sci 121:3677-82.

14. Balda, M. S., J. A. Whitney, C. Flores, S. Gonzalez, M. Cereijido, and K. Matter. 1996. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. J Cell Biol 134:1031-49.

15. Bamforth, S. D., U. Kniesel, H. Wolburg, B. Engelhardt, and W. Risau. 1999. A dominant mutant of occludin disrupts tight junction structure and function. J Cell Sci 112 (Pt 12):1879-88.

16. Bartenschlager, R., F. Penin, V. Lohmann, and P. Andre. 2011. Assembly of infectious hepatitis C virus particles. Trends Microbiol 19:95-103.

17. Barth, H., C. Schafer, M. I. Adah, F. Zhang, R. J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T. H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H. E. Blum, and T. F. Baumert. 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem 278:41003-12.

18. Barth, H., E. K. Schnober, F. Zhang, R. J. Linhardt, E. Depla, B. Boson, F. L. Cosset, A. H. Patel, H. E. Blum, and T. F. Baumert. 2006. Viral and cellular determinants of the hepatitis C virus envelope-heparan sulfate interaction. J Virol 80:10579-90.

19. Barton, E. S., J. C. Forrest, J. L. Connolly, J. D. Chappell, Y. Liu, F. J. Schnell, A. Nusrat, C. A. Parkos, and T. S. Dermody. 2001. Junction adhesion molecule is a receptor for reovirus. Cell 104:441-51.

20. Bartosch, B., J. Dubuisson, and F. L. Cosset. 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 197:633-42.

21. Bartosch, B., G. Verney, M. Dreux, P. Donot, Y. Morice, F. Penin, J. M. Pawlotsky, D. Lavillette, and F. L. Cosset. 2005. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J Virol 79:8217-29.

22. Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset. 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 278:41624-30.

23. Baumert, T. F., S. Ito, D. T. Wong, and T. J. Liang. 1998. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. J Virol 72:3827-36.

24. Beach, M. J., E. L. Meeks, L. T. Mimms, D. Vallari, L. DuCharme, J. Spelbring, S. Taskar, J. B. Schleicher, K. Krawczynski, and D. W. Bradley. 1992. Temporal relationships of hepatitis C virus RNA and antibody responses following experimental infection of chimpanzees. J Med Virol 36:226-37.

25. Benedicto, I., F. Molina-Jimenez, O. Barreiro, A. Maldonado-Rodriguez, J. Prieto, R. Moreno-Otero, R. Aldabe, M. Lopez-Cabrera, and P. L. Majano. 2008. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. Hepatology 48:1044-53.

26. Benedicto, I., F. Molina-Jimenez, B. Bartosch, F. L. Cosset, D. Lavillette, J. Prieto, R. Moreno-Otero, A. Valenzuela-Fernandez, R. Aldabe, M. Lopez-Cabrera, and P. L. Majano. 2009. The tight junction-associated protein occludin is required for a postbinding step in hepatitis C virus entry and infection. J Virol 83:8012-20.

27. Benga, W. J., S. E. Krieger, M. Dimitrova, M. B. Zeisel, M. Parnot, J. Lupberger, E. Hildt, G. Luo, J. McLauchlan, T. F. Baumert, and C. Schuster. 2010. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. Hepatology 51:43-53.

28. Bertaux, C., and T. Dragic. 2006. Different domains of CD81 mediate distinct stages of hepatitis C virus pseudoparticle entry. J Virol 80:4940-8.

29. Blanchard, E., S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski, and Y. Rouille. 2006. Hepatitis C virus entry depends on clathrin-mediated endocytosis. J Virol 80:6964-72.

30. Bradley, D., K. McCaustland, K. Krawczynski, J. Spelbring, C. Humphrey, and E. H. Cook. 1991. Hepatitis C virus: buoyant density of the factor VIII-derived isolate in sucrose. J Med Virol 34:206-8.

31. Branch, A. D., D. D. Stump, J. A. Gutierrez, F. Eng, and J. L. Walewski. 2005. The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. Semin Liver Dis 25:105-17.

32. Brazzoli, M., A. Bianchi, S. Filippini, A. Weiner, Q. Zhu, M. Pizza, and S. Crotta. 2008. CD81 is a central regulator of cellular events required for hepatitis C virus infection of human hepatocytes. J Virol 82:8316-29.

33. Brimacombe, C. L., J. Grove, L. W. Meredith, K. Hu, A. J. Syder, M. V. Flores, J. M. Timpe, S. E. Krieger, T. F. Baumert, T. L. Tellinghuisen, F. Wong-Staal, P. Balfe,

and J. A. McKeating. 2011. Neutralizing antibody-resistant hepatitis C virus cell-to-cell transmission. J Virol 85:596-605.

34. Burckhardt, C. J., and U. F. Greber. 2009. Virus movements on the plasma membrane support infection and transmission between cells. PLoS Pathog 5:e1000621.

35. Burgel, B., M. Friesland, A. Koch, M. P. Manns, H. Wedemeyer, K. Weissenborn, W. J. Schulz-Schaeffer, T. Pietschmann, E. Steinmann, and S. Ciesek. 2010. Hepatitis C virus enters human peripheral neuroblastoma cells - evidence for extra-hepatic cells sustaining hepatitis C virus penetration. J Viral Hepat. En prensa.

36. Burlone, M. E., and A. Budkowska. 2009. Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. J Gen Virol 90:1055-70.

37. Cai, Z., L. Cai, J. Jiang, K. S. Chang, D. R. van der Westhuyzen, and G. Luo. 2007. Human serum amyloid A protein inhibits hepatitis C virus entry into cells. J Virol 81:6128-33.

38. Catanese, M. T., H. Ansuini, R. Graziani, T. Huby, M. Moreau, J. K. Ball, G. Paonessa, C. M. Rice, R. Cortese, A. Vitelli, and A. Nicosia. 2010. Role of scavenger receptor class B type I in hepatitis C virus entry: kinetics and molecular determinants. J Virol 84:34-43.

39. Catanese, M. T., R. Graziani, T. von Hahn, M. Moreau, T. Huby, G. Paonessa, C. Santini, A. Luzzago, C. M. Rice, R. Cortese, A. Vitelli, and A. Nicosia. 2007. High-avidity monoclonal antibodies against the human scavenger class B type I receptor efficiently block hepatitis C virus infection in the presence of high-density lipoprotein. J Virol 81:8063-71.

40. Clayton, R. F., A. Owsianka, J. Aitken, S. Graham, D. Bhella, and A. H. Patel. 2002. Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. J Virol 76:7672-82.

41. Coller, K. E., K. L. Berger, N. S. Heaton, J. D. Cooper, R. Yoon, and G. Randall. 2009. RNA interference and single particle tracking analysis of hepatitis C virus endocytosis. PLoS Pathog 5:e1000702.

42. Connelly, M. A., and D. L. Williams. 2004. Scavenger receptor BI: a scavenger receptor with a mission to transport high density lipoprotein lipids. Curr Opin Lipidol 15:287-95.

43. Cormier, E. G., R. J. Durso, F. Tsamis, L. Boussemart, C. Manix, W. C. Olson, J. P. Gardner, and T. Dragic. 2004. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. Proc Natl Acad Sci U S A 101:14067-72.

44. Coyne, C. B., and J. M. Bergelson. 2006. Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. Cell 124:119-31.

45. Coyne, C. B., L. Shen, J. R. Turner, and J. M. Bergelson. 2007. Coxsackievirus entry across epithelial tight junctions requires occludin and the small GTPases Rab34 and Rab5. Cell Host Microbe 2:181-92.

46. Cukierman, L., L. Meertens, C. Bertaux, F. Kajumo, and T. Dragic. 2009. Residues in a highly conserved claudin-1 motif are required for hepatitis C virus entry and mediate the formation of cell-cell contacts. J Virol 83:5477-84.

47. Chang, K. S., J. Jiang, Z. Cai, and G. Luo. 2007. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. J Virol 81:13783-93.

48. Charrin, S., F. le Naour, O. Silvie, P. E. Milhiet, C. Boucheix, and E. Rubinstein. 2009. Lateral organization of membrane proteins: tetraspanins spin their web. Biochem J 420:133-54.

49. Chen, Y., C. Merzdorf, D. L. Paul, and D. A. Goodenough. 1997. COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos. J Cell Biol 138:891-9.

50. Decaens, C., M. Durand, B. Grosse, and D. Cassio. 2008. Which in vitro models could be best used to study hepatocyte polarity? Biol Cell 100:387-98.

51. Deforges, S., A. Evlashev, M. Perret, M. Sodoyer, S. Pouzol, J. Y. Scoazec, B. Bonnaud, O. Diaz, G. Paranhos-Baccala, V. Lotteau, and P. Andre. 2004. Expression of hepatitis C virus proteins in epithelial intestinal cells in vivo. J Gen Virol 85:2515-23.

52. Dhillon, S., J. Witteveldt, D. Gatherer, A. M. Owsianka, M. B. Zeisel, M. N. Zahid, M. Rychlowska, S. K. Foung, T. F. Baumert, A. G. Angus, and A. H. Patel. 2010. Mutations within a conserved region of the hepatitis C virus E2 glycoprotein that influence virus-receptor interactions and sensitivity to neutralizing antibodies. J Virol 84:5494-507.

53. Diaz, O., F. Delers, M. Maynard, S. Demignot, F. Zoulim, J. Chambaz, C. Trepo, V. Lotteau, and P. Andre. 2006. Preferential association of Hepatitis C virus with apolipoprotein B48-containing lipoproteins. J Gen Virol 87:2983-91.

54. Doughty, A. L., J. D. Spencer, Y. E. Cossart, and G. W. McCaughan. 1998. Cholestatic hepatitis after liver transplantation is associated with persistently high serum hepatitis C virus RNA levels. Liver Transpl Surg 4:15-21.

55. Dreux, M., V. L. Dao Thi, J. Fresquet, M. Guerin, Z. Julia, G. Verney, D. Durantel, F. Zoulim, D. Lavillette, F. L. Cosset, and B. Bartosch. 2009. Receptor complementation and mutagenesis reveal SR-BI as an essential HCV entry factor and functionally imply its intra- and extra-cellular domains. PLoS Pathog 5:e1000310.

56. Dubuisson, J. 2007. Hepatitis C virus proteins. World J Gastroenterol 13:2406-15.

57. Eckert, D. M., and P. S. Kim. 2001. Mechanisms of viral membrane fusion and its inhibition. Annu Rev Biochem 70:777-810.

58. Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol 76:5974-84.

59. Evans, M. J., T. von Hahn, D. M. Tscherne, A. J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J. A. McKeating, P. D. Bieniasz, and C. M. Rice. 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446:801-5.

60. Eyre, N. S., H. E. Drummer, and M. R. Beard. 2010. The SR-BI partner PDZK1 facilitates hepatitis C virus entry. PLoS Pathog 6.

61. Fanning, A. S., B. J. Jameson, L. A. Jesaitis, and J. M. Anderson. 1998. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem 273:29745-53.

62. Feldman, G. J., J. M. Mullin, and M. P. Ryan. 2005. Occludin: structure, function and regulation. Adv Drug Deliv Rev 57:883-917.

63. Felmlee, D. J., D. A. Sheridan, S. H. Bridge, S. U. Nielsen, R. W. Milne, C. J. Packard, M. J. Caslake, J. McLauchlan, G. L. Toms, R. D. Neely, and M. F. Bassendine. 2010. Intravascular transfer contributes to postprandial increase in numbers of very-low-density hepatitis C virus particles. Gastroenterology 139:1774-83.

64. Fletcher, N. F., J. P. Yang, M. J. Farquhar, K. Hu, C. Davis, Q. He, K. Dowd, S. C. Ray, S. E. Krieger, J. Neyts, T. F. Baumert, P. Balfe, J. A. McKeating, and F. Wong-Staal. 2010. Hepatitis C virus infection of neuroepithelioma cell lines. Gastroenterology 139:1365-74.

65. Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating. 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J Virol 73:6235-44.

66. Flint, M., T. von Hahn, J. Zhang, M. Farquhar, C. T. Jones, P. Balfe, C. M. Rice, and J. A. McKeating. 2006. Diverse CD81 proteins support hepatitis C virus infection. J Virol 80:11331-42.

67. Forton, D. M., P. Karayiannis, N. Mahmud, S. D. Taylor-Robinson, and H. C. Thomas. 2004. Identification of unique hepatitis C virus quasispecies in the central nervous system and comparative analysis of internal translational efficiency of brain, liver, and serum variants. J Virol 78:5170-83.

68. Fournier, C., C. Sureau, J. Coste, J. Ducos, G. Pageaux, D. Larrey, J. Domergue, and P. Maurel. 1998. In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. J Gen Virol 79 (Pt 10):2367-74.

69. Fujimoto, K. 1995. Freeze-fracture replica electron microscopy combined with SDS digestion for cytochemical labeling of integral membrane proteins. Application to the immunogold labeling of intercellular junctional complexes. J Cell Sci 108 (Pt 11):3443-9.

70. Furuse, M. Molecular basis of the core structure of tight junctions. 2010. Cold Spring Harb Perspect Biol 2:a002907.

71. Furuse, M., M. Hata, K. Furuse, Y. Yoshida, A. Haratake, Y. Sugitani, T. Noda, A. Kubo, and S. Tsukita. 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156:1099-111.

72. Furuse, M., T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, and S. Tsukita. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 123:1777-88.

73. Furuse, M., M. Itoh, T. Hirase, A. Nagafuchi, S. Yonemura, and S. Tsukita. 1994. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J Cell Biol 127:1617-26.

74. Furuse, M., H. Sasaki, K. Fujimoto, and S. Tsukita. 1998. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 143:391-401.

75. Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson. 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc Natl Acad Sci U S A 100:4498-503.

76. Gastaminza, P., G. Cheng, S. Wieland, J. Zhong, W. Liao, and F. V. Chisari. 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. J Virol 82:2120-9.

77. Gastaminza, P., S. B. Kapadia, and F. V. Chisari. 2006. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. J Virol 80:11074-81.

78. Gelman, M. A., and J. S. Glenn. 2010 Mixing the right hepatitis C inhibitor cocktail. Trends Mol Med. En prensa.

79. Ghassemifar, M. R., B. Sheth, T. Papenbrock, H. J. Leese, F. D. Houghton, and T. P. Fleming. 2002. Occludin TM4(-): an isoform of the tight junction protein present in primates lacking the fourth transmembrane domain. J Cell Sci 115:3171-80.

80. Ginsberg, H. N., and E. A. Fisher. 2009. The ever-expanding role of degradation in the regulation of apolipoprotein B metabolism. J Lipid Res 50 Suppl:S162-6.

81. Goffard, A., N. Callens, B. Bartosch, C. Wychowski, F. L. Cosset, C. Montpellier, and J. Dubuisson. 2005. Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. J Virol 79:8400-9.

82. Gondeau, C., L. Pichard-Garcia, and P. Maurel. 2009. Cellular models for the screening and development of anti-hepatitis C virus agents. Pharmacol Ther 124:1-22.

83. Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B. E. Jaramillo. 2003. Tight junction proteins. Prog Biophys Mol Biol 81:1-44.

84. Gonzalez-Mariscal, L., E. Garay, and S. Lechuga. 2009. Virus interaction with the apical junctional complex. Front Biosci 14:731-68.

85. Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour. 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J Virol 77:5487-92.

86. Gottwein, J. M., T. K. Scheel, A. M. Hoegh, J. B. Lademann, J. Eugen-Olsen, G. Lisby, and J. Bukh. 2007. Robust hepatitis C genotype 3a cell culture releasing adapted intergenotypic 3a/2a (S52/JFH1) viruses. Gastroenterology 133:1614-26.

87. Gottwein, J. M., T. K. Scheel, T. B. Jensen, J. B. Lademann, J. C. Prentoe, M. L. Knudsen, A. M. Hoegh, and J. Bukh. 2009. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. Hepatology 49:364-77.

88. Grove, J., T. Huby, Z. Stamataki, T. Vanwolleghem, P. Meuleman, M. Farquhar, A. Schwarz, M. Moreau, J. S. Owen, G. Leroux-Roels, P. Balfe, and J. A. McKeating. 2007. Scavenger receptor BI and BII expression levels modulate hepatitis C virus infectivity. J Virol 81:3162-9.

89. Guidotti, L. G., and F. V. Chisari. 2006. Immunobiology and pathogenesis of viral hepatitis. Annu Rev Pathol 1:23-61.

90. Guttman, J. A., and B. B. Finlay. 2009. Tight junctions as targets of infectious agents. Biochim Biophys Acta 1788:832-41.

91. Haid, S., T. Pietschmann, and E. I. Pecheur. 2009. Low pH-dependent hepatitis C virus membrane fusion depends on E2 integrity, target lipid composition, and density of virus particles. J Biol Chem 284:17657-67.

92. Harris, H. J., C. Davis, J. G. Mullins, K. Hu, M. Goodall, M. J. Farquhar, C. J. Mee, K. McCaffrey, S. Young, H. Drummer, P. Balfe, and J. A. McKeating. 2010. Claudin association with CD81 defines hepatitis C virus entry. J Biol Chem 285:21092-102.

93. Harris, H. J., M. J. Farquhar, C. J. Mee, C. Davis, G. M. Reynolds, A. Jennings, K. Hu, F. Yuan, H. Deng, S. G. Hubscher, J. H. Han, P. Balfe, and J. A. McKeating. 2008. CD81 and claudin 1 coreceptor association: role in hepatitis C virus entry. J Virol 82:5007-20.

94. Haussecker, D., and M. A. Kay. 2010. miR-122 continues to blaze the trail for microRNA therapeutics. Mol Ther 18:240-2.

95. Helle, F., and J. Dubuisson. 2008. Hepatitis C virus entry into host cells. Cell Mol Life Sci 65:100-12.

96. Helle, F., A. Goffard, V. Morel, G. Duverlie, J. McKeating, Z. Y. Keck, S. Foung, F. Penin, J. Dubuisson, and C. Voisset. 2007. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. J Virol 81:8101-11.

97. Hijikata, M., Y. K. Shimizu, H. Kato, A. Iwamoto, J. W. Shih, H. J. Alter, R. H. Purcell, and H. Yoshikura. 1993. Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. J Virol 67:1953-8.

98. Hoofnagle, J. H. 2002. Course and outcome of hepatitis C. Hepatology 36:S21-9.

99. Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating. 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 100:7271-6.

100. Huang, H., F. Sun, D. M. Owen, W. Li, Y. Chen, M. Gale, Jr., and J. Ye. 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc Natl Acad Sci U S A 104:5848-53.

101. Icard, V., O. Diaz, C. Scholtes, L. Perrin-Cocon, C. Ramiere, R. Bartenschlager, F. Penin, V. Lotteau, and P. Andre. 2009. Secretion of hepatitis C virus envelope glycoproteins depends on assembly of apolipoprotein B positive lipoproteins. PLoS One 4:e4233.

102. Jensen, T. B., J. M. Gottwein, T. K. Scheel, A. M. Hoegh, J. Eugen-Olsen, and J. Bukh. 2008. Highly efficient JFH1-based cell-culture system for hepatitis C virus genotype 5a: failure of homologous neutralizing-antibody treatment to control infection. J Infect Dis 198:1756-65.

103. Jiang, J., and G. Luo. 2009. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J Virol 83:12680-91.

104. Jones, D. M., and J. McLauchlan. 2010. Hepatitis C virus: assembly and release of virus particles. J Biol Chem 285:22733-9.

105. Kaito, M., S. Watanabe, K. Tsukiyama-Kohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishida, S. Suzuki, and M. Kohara. 1994. Hepatitis C virus particle detected by immunoelectron microscopic study. J Gen Virol 75 (Pt 7):1755-60.

106. Kapadia, S. B., H. Barth, T. Baumert, J. A. McKeating, and F. V. Chisari. 2007. Initiation of hepatitis C virus infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J Virol 81:374-83.

107. Kielian, M., and F. A. Rey. 2006. Virus membrane-fusion proteins: more than one way to make a hairpin. Nat Rev Microbiol 4:67-76.

108. Kleinman, H. K., and G. R. Martin. 2005. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol 15:378-86.

109. Kobayashi, M., M. C. Bennett, T. Bercot, and I. R. Singh. 2006. Functional analysis of hepatitis C virus envelope proteins, using a cell-cell fusion assay. J Virol 80:1817-25.

110. Kohaar, I., A. Ploss, E. Korol, K. Mu, J. W. Schoggins, T. R. O'Brien, C. M. Rice, and L. Prokunina-Olsson. 2010. Splicing diversity of the human OCLN gene and its biological significance for hepatitis C virus entry. J Virol 84:6987-94.

111. Kohler, K., and A. Zahraoui. 2005. Tight junction: a co-ordinator of cell signalling and membrane trafficking. Biol Cell 97:659-65.

112. Korn, W. M., M. Macal, C. Christian, M. D. Lacher, A. McMillan, K. A. Rauen, R. S. Warren, and L. Ferrell. 2006. Expression of the coxsackievirus- and adenovirus receptor in gastrointestinal cancer correlates with tumor differentiation. Cancer Gene Ther 13:792-7.

113. Koutsoudakis, G., E. Herrmann, S. Kallis, R. Bartenschlager, and T. Pietschmann. 2007. The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. J Virol 81:588-98.

114. Koutsoudakis, G., A. Kaul, E. Steinmann, S. Kallis, V. Lohmann, T. Pietschmann, and R. Bartenschlager. 2006. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J Virol 80:5308-20.

115. Kovalenko, O. V., X. H. Yang, and M. E. Hemler. 2007. A novel cysteine cross-linking method reveals a direct association between claudin-1 and tetraspanin CD9. Mol Cell Proteomics 6:1855-67.

116. Krieger, S. E., M. B. Zeisel, C. Davis, C. Thumann, H. J. Harris, E. K. Schnober, C. Mee, E. Soulier, C. Royer, M. Lambotin, F. Grunert, V. L. Dao Thi, M. Dreux, F. L. Cosset, J. A. McKeating, C. Schuster, and T. F. Baumert. 2010. Inhibition of hepatitis C virus infection by anti-claudin-1 antibodies is mediated by neutralization of E2-CD81-claudin-1 associations. Hepatology 51:1144-57.

117. Lacaz-Vieira, F., M. M. Jaeger, P. Farshori, and B. Kachar. 1999. Small synthetic peptides homologous to segments of the first external loop of occludin impair tight junction resealing. J Membr Biol 168:289-97.

118. Lanford, R. E., D. Chavez, F. V. Chisari, and C. Sureau. 1995. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 69:8079-83.

119. Lanford, R. E., E. S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M. E. Munk, S. Kauppinen, and H. Orum. 2010. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327:198-201.

120. Lavie, M., C. Voisset, N. Vu-Dac, V. Zurawski, G. Duverlie, C. Wychowski, and J. Dubuisson. 2006. Serum amyloid A has antiviral activity against hepatitis C virus by inhibiting virus entry in a cell culture system. Hepatology 44:1626-34.

121. Lavillette, D., B. Bartosch, D. Nourrisson, G. Verney, F. L. Cosset, F. Penin, and E. I. Pecheur. 2006. Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes. J Biol Chem 281:3909-17.

122. Lavillette, D., E. I. Pecheur, P. Donot, J. Fresquet, J. Molle, R. Corbau, M. Dreux, F. Penin, and F. L. Cosset. 2007. Characterization of fusion determinants points to the involvement of three discrete regions of both E1 and E2 glycoproteins in the membrane fusion process of hepatitis C virus. J Virol 81:8752-65.

123. Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset. 2005. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology 41:265-74.

124. Li, X., L. J. Jeffers, L. Shao, K. R. Reddy, M. de Medina, J. Scheffel, B. Moore, and E. R. Schiff. 1995. Identification of hepatitis C virus by immunoelectron microscopy. J Viral Hepat 2:227-34.

125. Liang, Y., T. Shilagard, S. Y. Xiao, N. Snyder, D. Lau, L. Cicalese, H. Weiss, G. Vargas, and S. M. Lemon. 2009. Visualizing hepatitis C virus infections in human liver by two-photon microscopy. Gastroenterology 137:1448-58.

126. Lindenbach, B. D. 2010. New cell culture models of hepatitis C virus entry, replication, and virus production. Gastroenterology 139:1090-3.

127. Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice. 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623-6.

128. Lindenbach, B. D., P. Meuleman, A. Ploss, T. Vanwolleghem, A. J. Syder, J. A. McKeating, R. E. Lanford, S. M. Feinstone, M. E. Major, G. Leroux-Roels, and C. M. Rice. 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc Natl Acad Sci U S A 103:3805-9.

129. Liu, S., W. Kuo, W. Yang, W. Liu, G. A. Gibson, K. Dorko, S. C. Watkins, S. C. Strom, and T. Wang. 2010. The second extracellular loop dictates Occludin-mediated HCV entry. Virology 407:160-70.

130. Liu, S., W. Yang, L. Shen, J. R. Turner, C. B. Coyne, and T. Wang. 2009. Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. J Virol 83:2011-4.

131. Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110-3.

132. Lozach, P. Y., A. Amara, B. Bartosch, J. L. Virelizier, F. Arenzana-Seisdedos, F. L. Cosset, and R. Altmeyer. 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. J Biol Chem 279:32035-45.

133. Lozach, P. Y., H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer. 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J Biol Chem 278:20358-66.

134. Lui, W. Y., and W. M. Lee. 2005. cAMP perturbs inter-Sertoli tight junction permeability barrier in vitro via its effect on proteasome-sensitive ubiquitination of occludin. J Cell Physiol 203:564-72.

135. Maillard, P., T. Huby, U. Andreo, M. Moreau, J. Chapman, and A. Budkowska. 2006. The interaction of natural hepatitis C virus with human scavenger receptor SR-BI/Cla1 is mediated by ApoB-containing lipoproteins. FASEB J 20:735-7.

136. Maillard, P., K. Krawczynski, J. Nitkiewicz, C. Bronnert, M. Sidorkiewicz, P. Gounon, J. Dubuisson, G. Faure, R. Crainic, and A. Budkowska. 2001. Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. J Virol 75:8240-50.

137. Makino, A., M. Shimojima, T. Miyazawa, K. Kato, Y. Tohya, and H. Akashi. 2006. Junctional adhesion molecule 1 is a functional receptor for feline calicivirus. J Virol 80:4482-90.

138. Mankertz, J., J. S. Waller, B. Hillenbrand, S. Tavalali, P. Florian, T. Schoneberg, M. Fromm, and J. D. Schulzke. 2002. Gene expression of the tight junction protein occludin includes differential splicing and alternative promoter usage. Biochem Biophys Res Commun 298:657-66.

139. Marukian, S., C. T. Jones, L. Andrus, M. J. Evans, K. D. Ritola, E. D. Charles, C. M. Rice, and L. B. Dustin. 2008. Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatology 48:1843-50.

140. Masciopinto, F., C. Giovani, S. Campagnoli, L. Galli-Stampino, P. Colombatto, M. Brunetto, T. S. Yen, M. Houghton, P. Pileri, and S. Abrignani. 2004. Association of hepatitis C virus envelope proteins with exosomes. Eur J Immunol 34:2834-42.

141. Matter, K., and M. S. Balda. 1998. Biogenesis of tight junctions: the C-terminal domain of occludin mediates basolateral targeting. J Cell Sci 111 (Pt 4):511-9.

142. Matter, K., and M. S. Balda. 2003. Signalling to and from tight junctions. Nat Rev Mol Cell Biol 4:225-36.

143. McCarthy, J. J., J. H. Li, A. Thompson, S. Suchindran, X. Q. Lao, K. Patel, H. L. Tillmann, A. J. Muir, and J. G. McHutchison. 2010. Replicated association between an IL28B gene variant and a sustained response to pegylated interferon and ribavirin. Gastroenterology 138:2307-14.

144. McCarthy, K. M., I. B. Skare, M. C. Stankewich, M. Furuse, S. Tsukita, R. A. Rogers, R. D. Lynch, and E. E. Schneeberger. 1996. Occludin is a functional component of the tight junction. J Cell Sci 109 (Pt 9):2287-98.

145. Medina, R., C. Rahner, L. L. Mitic, J. M. Anderson, and C. M. Van Itallie. 2000. Occludin localization at the tight junction requires the second extracellular loop. J Membr Biol 178:235-47.

146. Mee, C. J., M. J. Farquhar, H. J. Harris, K. Hu, W. Ramma, A. Ahmed, P. Maurel, R. Bicknell, P. Balfe, and J. A. McKeating. 2010. Hepatitis C virus infection reduces hepatocellular polarity in a vascular endothelial growth factor-dependent manner. Gastroenterology 138:1134-42.

147. Mee, C. J., J. Grove, H. J. Harris, K. Hu, P. Balfe, and J. A. McKeating. 2008. Effect of cell polarization on hepatitis C virus entry. J Virol 82:461-70.

148. Mee, C. J., H. J. Harris, M. J. Farquhar, G. Wilson, G. Reynolds, C. Davis, I. S. C. van, P. Balfe, and J. A. McKeating. 2009. Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells. J Virol 83:6211-21.

149. Meertens, L., C. Bertaux, L. Cukierman, E. Cormier, D. Lavillette, F. L. Cosset, and T. Dragic. 2008. The tight junction proteins claudin-1, -6, and -9 are entry cofactors for hepatitis C virus. J Virol 82:3555-60.

150. Meertens, L., C. Bertaux, and T. Dragic. 2006. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. J Virol 80:11571-8.

151. Mensa, L., G. Crespo, M. J. Gastinger, J. Kabat, S. Perez-Del-Pulgar, R. Miquel, S. U. Emerson, R. H. Purcell, and X. Forns. 2010. Hepatitis C virus receptors claudin-1 and occludin after liver transplantation and influence on early viral kinetics. Hepatology. En prensa.

152. Meuleman, P., J. Hesselgesser, M. Paulson, T. Vanwolleghem, I. Desombere, H. Reiser, and G. Leroux-Roels. 2008. Anti-CD81 antibodies can prevent a hepatitis C virus infection in vivo. Hepatology 48:1761-8.

153. Meunier, J. C., R. S. Russell, R. E. Engle, K. N. Faulk, R. H. Purcell, and S. U. Emerson. 2008. Apolipoprotein c1 association with hepatitis C virus. J Virol 82:9647-56.

154. Michta, M. L., S. E. Hopcraft, C. M. Narbus, Z. Kratovac, B. Israelow, M. Sourisseau, and M. J. Evans. 2010. Species-specific regions of occludin required by hepatitis C virus for cell entry. J Virol 84:11696-708.

155. Molina, S., V. Castet, C. Fournier-Wirth, L. Pichard-Garcia, R. Avner, D. Harats, J. Roitelman, R. Barbaras, P. Graber, P. Ghersa, M. Smolarsky, A. Funaro, F. Malavasi, D. Larrey, J. Coste, J. M. Fabre, A. Sa-Cunha, and P. Maurel. 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J Hepatol 46:411-9.

156. Molina, S., V. Castet, L. Pichard-Garcia, C. Wychowski, E. Meurs, J. M. Pascussi, C. Sureau, J. M. Fabre, A. Sacunha, D. Larrey, J. Dubuisson, J. Coste, J. McKeating, P. Maurel, and C. Fournier-Wirth. 2008. Serum-derived hepatitis C virus infection of primary human hepatocytes is tetraspanin CD81 dependent. J Virol 82:569-74.

157. Morimoto, S., N. Nishimura, T. Terai, S. Manabe, Y. Yamamoto, W. Shinahara, H. Miyake, S. Tashiro, M. Shimada, and T. Sasaki. 2005. Rab13 mediates the continuous endocytic recycling of occludin to the cell surface. J Biol Chem 280:2220-8.

158. Murakami, K., K. Ishii, Y. Ishihara, S. Yoshizaki, K. Tanaka, Y. Gotoh, H. Aizaki, M. Kohara, H. Yoshioka, Y. Mori, N. Manabe, I. Shoji, T. Sata, R. Bartenschlager, Y. Matsuura, T. Miyamura, and T. Suzuki. 2006. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 351:381-92.

159. Murakami, T., E. A. Felinski, and D. A. Antonetti. 2009. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. J Biol Chem 284:21036-46.

160. Muresan, Z., D. L. Paul, and D. A. Goodenough. 2000. Occludin 1B, a variant of the tight junction protein occludin. Mol Biol Cell 11:627-34.

161. Nava, P., S. Lopez, C. F. Arias, S. Islas, and L. Gonzalez-Mariscal. 2004. The rotavirus surface protein VP8 modulates the gate and fence function of tight junctions in epithelial cells. J Cell Sci 117:5509-19.

162. Nielsen, S. U., M. F. Bassendine, A. D. Burt, C. Martin, W. Pumeechockchai, and G. L. Toms. 2006. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. J Virol 80:2418-28.

163. Orban, E., E. Szabo, G. Lotz, P. Kupcsulik, C. Paska, Z. Schaff, and A. Kiss. 2008. Different expression of occludin and ZO-1 in primary and metastatic liver tumors. Pathol Oncol Res 14:299-306.

164. Ouban, A., and A. A. Ahmed. 2010. Claudins in human cancer: a review. Histol Histopathol 25:83-90.

165. Owen, D. M., H. Huang, J. Ye, and M. Gale, Jr. 2009. Apolipoprotein E on hepatitis C virion facilitates infection through interaction with low-density lipoprotein receptor. Virology 394:99-108.

166. Owsianka, A., R. F. Clayton, L. D. Loomis-Price, J. A. McKeating, and A. H. Patel. 2001. Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. J Gen Virol 82:1877-83.

167. Owsianka, A. M., J. M. Timms, A. W. Tarr, R. J. Brown, T. P. Hickling, A. Szwejk, K. Bienkowska-Szewczyk, B. J. Thomson, A. H. Patel, and J. K. Ball. 2006. Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. J Virol 80:8695-704.

168. Patel, A. H., J. Wood, F. Penin, J. Dubuisson, and J. A. McKeating. 2000. Construction and characterization of chimeric hepatitis C virus E2 glycoproteins: analysis of regions critical for glycoprotein aggregation and CD81 binding. J Gen Virol 81:2873-83.

169. Penin, F., J. Dubuisson, F. A. Rey, D. Moradpour, and J. M. Pawlotsky. 2004. Structural biology of hepatitis C virus. Hepatology 39:5-19.

170. Perrault, M., and E. I. Pecheur. 2009. The hepatitis C virus and its hepatic environment: a toxic but finely tuned partnership. Biochem J 423:303-14.

171. Pietschmann, T., V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand, and R. Bartenschlager. 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. J Virol 76:4008-21.

172. Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani. 1998. Binding of hepatitis C virus to CD81. Science 282:938-41.

173. Ploss, A., M. J. Evans, V. A. Gaysinskaya, M. Panis, H. You, Y. P. de Jong, and C. M. Rice. 2009. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457:882-6.

174. Ploss, A., and C. M. Rice. 2009. Towards a small animal model for hepatitis C. EMBO Rep 10:1220-7.

175. Podevin, P., A. Carpentier, V. Pene, L. Aoudjehane, M. Carriere, S. Zaidi, C. Hernandez, V. Calle, J. F. Meritet, O. Scatton, M. Dreux, F. L. Cosset, T. Wakita, R. Bartenschlager, S. Demignot, F. Conti, A. R. Rosenberg, and Y. Calmus. 2010. Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. Gastroenterology 139:1355-64.

176. Poynard, T., M. F. Yuen, V. Ratziu, and C. L. Lai. 2003. Viral hepatitis C. Lancet 362:2095-100.

177. Prince, A. M., T. Huima-Byron, T. S. Parker, and D. M. Levine. 1996. Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. J Viral Hepat 3:11-7.

178. Rahner, C., B. Stieger, and L. Landmann. 1996. Structure-function correlation of tight junctional impairment after intrahepatic and extrahepatic cholestasis in rat liver. Gastroenterology 110:1564-78.

179. Raleigh, D. R., A. M. Marchiando, Y. Zhang, L. Shen, H. Sasaki, Y. Wang, M. Long, and J. R. Turner. 2010. Tight junction-associated MARVEL proteins marveld3, tricellulin, and occludin have distinct but overlapping functions. Mol Biol Cell 21:1200-13.

180. Ratcliffe, D. R., J. Iqbal, M. M. Hussain, and E. B. Cramer. 2009. Fibrillar collagen type I stimulation of apolipoprotein B secretion in Caco-2 cells is mediated by beta1 integrin. Biochim Biophys Acta 1791:1144-54.

181. Reynolds, G. M., H. J. Harris, A. Jennings, K. Hu, J. Grove, P. F. Lalor, D. H. Adams, P. Balfe, S. G. Hubscher, and J. A. McKeating. 2008. Hepatitis C virus receptor expression in normal and diseased liver tissue. Hepatology 47:418-27.

182. Rigotti, A., H. E. Miettinen, and M. Krieger. 2003. The role of the high-density lipoprotein receptor SR-BI in the lipid metabolism of endocrine and other tissues. Endocr Rev 24:357-87.

183. Rocha-Perugini, V., M. Lavie, D. Delgrange, J. Canton, A. Pillez, J. Potel, C. Lecoeur, E. Rubinstein, J. Dubuisson, C. Wychowski, and L. Cocquerel. 2009. The association of CD81 with tetraspanin-enriched microdomains is not essential for Hepatitis C virus entry. BMC Microbiol 9:111.

184. Rocha-Perugini, V., C. Montpellier, D. Delgrange, C. Wychowski, F. Helle, A. Pillez, H. Drobecq, F. Le Naour, S. Charrin, S. Levy, E. Rubinstein, J. Dubuisson, and L. Cocquerel. 2008. The CD81 partner EWI-2wint inhibits hepatitis C virus entry. PLoS One 3:e1866.

185. Roohvand, F., P. Maillard, J. P. Lavergne, S. Boulant, M. Walic, U. Andreo, L. Goueslain, F. Helle, A. Mallet, J. McLauchlan, and A. Budkowska. 2009. Initiation of hepatitis C virus infection requires the dynamic microtubule network: role of the viral nucleocapsid protein. J Biol Chem 284:13778-91.

186. Rumin, S., P. Berthillon, E. Tanaka, K. Kiyosawa, M. A. Trabaud, T. Bizollon, C. Gouillat, P. Gripon, C. Guguen-Guillouzo, G. Inchauspe, and C. Trepo. 1999. Dynamic analysis of hepatitis C virus replication and quasispecies selection in long-term cultures of adult human hepatocytes infected in vitro. J Gen Virol 80 (Pt 11):3007-18.

187. Sainz, B., Jr., V. TenCate, and S. L. Uprichard. 2009. Three-dimensional Huh7 cell culture system for the study of Hepatitis C virus infection. Virol J 6:103.

188. Saitou, M., M. Furuse, H. Sasaki, J. D. Schulzke, M. Fromm, H. Takano, T. Noda, and S. Tsukita. 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 11:4131-42.

189. Sakisaka, S., T. Kawaguchi, E. Taniguchi, S. Hanada, K. Sasatomi, H. Koga, M. Harada, R. Kimura, M. Sata, N. Sawada, M. Mori, S. Todo, and T. Kurohiji. 2001. Alterations in tight junctions differ between primary biliary cirrhosis and primary sclerosing cholangitis. Hepatology 33:1460-8.

190. Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli. 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 21:5017-25.

191. Scheel, T. K., J. M. Gottwein, T. B. Jensen, J. C. Prentoe, A. M. Hoegh, H. J. Alter, J. Eugen-Olsen, and J. Bukh. 2008. Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. Proc Natl Acad Sci U S A 105:997-1002.

192. Schmitt, M., A. Horbach, R. Kubitz, A. Frilling, and D. Haussinger. 2004. Disruption of hepatocellular tight junctions by vascular endothelial growth factor (VEGF): a novel mechanism for tumor invasion. J Hepatol 41:274-83.

193. Schulzke, J. D., A. H. Gitter, J. Mankertz, S. Spiegel, U. Seidler, S. Amasheh, M. Saitou, S. Tsukita, and M. Fromm. 2005. Epithelial transport and barrier function in occludin-deficient mice. Biochim Biophys Acta 1669:34-42.

194. Schwarz, A. K., J. Grove, K. Hu, C. J. Mee, P. Balfe, and J. A. McKeating. 2009. Hepatoma cell density promotes claudin-1 and scavenger receptor BI expression and hepatitis C virus internalization. J Virol 83:12407-14.

195. Shen, L., C. R. Weber, and J. R. Turner. 2008. The tight junction protein complex undergoes rapid and continuous molecular remodeling at steady state. J Cell Biol 181:683-95.

196. Shimizu, Y., T. Hishiki, K. Sugiyama, K. Ogawa, K. Funami, A. Kato, Y. Ohsaki, T. Fujimoto, H. Takaku, and K. Shimotohno. 2010. Lipoprotein lipase and hepatic triglyceride lipase reduce the infectivity of hepatitis C virus (HCV) through their catalytic activities on HCV-associated lipoproteins. Virology 407:152-9.

197. Silvie, O., E. Rubinstein, J. F. Franetich, M. Prenant, E. Belnoue, L. Renia, L. Hannoun, W. Eling, S. Levy, C. Boucheix, and D. Mazier. 2003. Hepatocyte CD81 is required for Plasmodium falciparum and Plasmodium yoelii sporozoite infectivity. Nat Med 9:93-6.

198. Stamataki, Z., C. Shannon-Lowe, J. Shaw, D. Mutimer, A. B. Rickinson, J. Gordon, D. H. Adams, P. Balfe, and J. A. McKeating. 2009. Hepatitis C virus association with peripheral blood B lymphocytes potentiates viral infection of liver-derived hepatoma cells. Blood 113:585-93.

199. Steed, E., M. S. Balda, and K. Matter. 2010. Dynamics and functions of tight junctions. Trends Cell Biol 20:142-9.

200. Steed, E., N. T. Rodrigues, M. S. Balda, and K. Matter. 2009. Identification of MarvelD3 as a tight junction-associated transmembrane protein of the occludin family. BMC Cell Biol 10:95.

201. Suppiah, V., M. Moldovan, G. Ahlenstiel, T. Berg, M. Weltman, M. L. Abate, M. Bassendine, U. Spengler, G. J. Dore, E. Powell, S. Riordan, D. Sheridan, A. Smedile, V. Fragomeli, T. Muller, M. Bahlo, G. J. Stewart, D. R. Booth, and J. George. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 41:1100-4.

202. Tanaka, Y., N. Nishida, M. Sugiyama, M. Kurosaki, K. Matsuura, N. Sakamoto, M. Nakagawa, M. Korenaga, K. Hino, S. Hige, Y. Ito, E. Mita, E. Tanaka, S. Mochida, Y. Murawaki, M. Honda, A. Sakai, Y. Hiasa, S. Nishiguchi, A. Koike, I. Sakaida, M. Imamura, K. Ito, K. Yano, N. Masaki, F. Sugauchi, N. Izumi, K. Tokunaga, and M. Mizokami. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 41:1105-9.

203. Timpe, J. M., Z. Stamataki, A. Jennings, K. Hu, M. J. Farquhar, H. J. Harris, A. Schwarz, I. Desombere, G. L. Roels, P. Balfe, and J. A. McKeating. 2008. Hepatitis C

virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. Hepatology 47:17-24.

204. Tong, Y., Y. Zhu, Y. Liu, Y. Feng, X. Hua, Z. Chen, H. Ding, L. Gao, Y. Wang, M. A. Feitelson, X. Xia, P. Zhao, and Z. Qi. 2011. Tupaia CD81, SR-BI, claudin-1, and occludin support hepatitis C virus infection. J Virol 85:2793-802.

205. Traber, M. G., H. J. Kayden, and M. J. Rindler. 1987. Polarized secretion of newly synthesized lipoproteins by the Caco-2 human intestinal cell line. J Lipid Res 28:1350-63.

206. Trauner, M., P. J. Meier, and J. L. Boyer. 1998. Molecular pathogenesis of cholestasis. N Engl J Med 339:1217-27.

207. Traweger, A., D. Fang, Y. C. Liu, W. Stelzhammer, I. A. Krizbai, F. Fresser, H. C. Bauer, and H. Bauer. 2002. The tight junction-specific protein occludin is a functional target of the E3 ubiquitin-protein ligase itch. J Biol Chem 277:10201-8.

208. Tscherne, D. M., C. T. Jones, M. J. Evans, B. D. Lindenbach, J. A. McKeating, and C. M. Rice. 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J Virol 80:1734-41.

209. Van Itallie, C. M., and J. M. Anderson. 2006. Claudins and epithelial paracellular transport. Annu Rev Physiol 68:403-29.

210. Van Itallie, C. M., and J. M. Anderson. 1997. Occludin confers adhesiveness when expressed in fibroblasts. J Cell Sci 110 (Pt 9):1113-21.

211. Vietor, I., T. Bader, K. Paiha, and L. A. Huber. 2001. Perturbation of the tight junction permeability barrier by occludin loop peptides activates beta-catenin/TCF/LEF-mediated transcription. EMBO Rep 2:306-12.

212. Vieyres, G., X. Thomas, V. Descamps, G. Duverlie, A. H. Patel, and J. Dubuisson. 2010. Characterization of the envelope glycoproteins associated with infectious hepatitis C virus. J Virol 84:10159-68.

213. Voisset, C., N. Callens, E. Blanchard, A. Op De Beeck, J. Dubuisson, and N. Vu-Dac. 2005. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. J Biol Chem 280:7793-9.

214. von Hahn, T., B. D. Lindenbach, A. Boullier, O. Quehenberger, M. Paulson, C. M. Rice, and J. A. McKeating. 2006. Oxidized low-density lipoprotein inhibits hepatitis C virus cell entry in human hepatoma cells. Hepatology 43:932-42.

215. von Hahn, T., and C. M. Rice. 2008. Hepatitis C virus entry. J Biol Chem 283:3689-93.

216. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.

2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11:791-6.

217. Walters, R. W., P. Freimuth, T. O. Moninger, I. Ganske, J. Zabner, and M. J. Welsh. 2002. Adenovirus fiber disrupts CAR-mediated intercellular adhesion allowing virus escape. Cell 110:789-99.

218. Washburn, M. L., M. T. Bility, L. Zhang, G. I. Kovalev, A. Buntzman, J. A. Frelinger, W. Barry, A. Ploss, C. M. Rice, and L. Su. 2011. A Humanized Mouse Model to Study Hepatitis C Virus Infection, Immune Response, and Liver Disease. Gastroenterology 140:1334-44.

219. Wilkinson, J., M. Radkowski, and T. Laskus. 2009. Hepatitis C virus neuroinvasion: identification of infected cells. J Virol 83:1312-9.

220. Witteveldt, J., M. J. Evans, J. Bitzegeio, G. Koutsoudakis, A. M. Owsianka, A. G. Angus, Z. Y. Keck, S. K. Foung, T. Pietschmann, C. M. Rice, and A. H. Patel. 2009. CD81 is dispensable for hepatitis C virus cell-to-cell transmission in hepatoma cells. J Gen Virol 90:48-58.

221. Wong, V., and B. M. Gumbiner. 1997. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J Cell Biol 136:399-409.

222. Yang, W., C. Qiu, N. Biswas, J. Jin, S. C. Watkins, R. C. Montelaro, C. B. Coyne, and T. Wang. 2008. Correlation of the tight junction-like distribution of Claudin-1 to the cellular tropism of hepatitis C virus. J Biol Chem 283:8643-53.

223. Yu, D., and J. R. Turner. 2008. Stimulus-induced reorganization of tight junction structure: the role of membrane traffic. Biochim Biophys Acta 1778:709-16.

224. Yu, X., M. Qiao, I. Atanasov, Z. Hu, T. Kato, T. J. Liang, and Z. H. Zhou. 2007. Cryo-electron microscopy and three-dimensional reconstructions of hepatitis C virus particles. Virology 367:126-34.

225. Zeisel, M. B., I. Fofana, S. Fafi-Kremer, and T. F. Baumert. 2011. Hepatitis C virus entry into hepatocytes: Molecular mechanisms and targets for antiviral therapies. J Hepatol 54:566-76.

226. Zeisel, M. B., G. Koutsoudakis, E. K. Schnober, A. Haberstroh, H. E. Blum, F. L. Cosset, T. Wakita, D. Jaeck, M. Doffoel, C. Royer, E. Soulier, E. Schvoerer, C. Schuster, F. Stoll-Keller, R. Bartenschlager, T. Pietschmann, H. Barth, and T. F. Baumert. 2007. Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. Hepatology 46:1722-31.

227. Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating. 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. J Virol 78:1448-55.

228. Zheng, A., F. Yuan, Y. Li, F. Zhu, P. Hou, J. Li, X. Song, M. Ding, and H. Deng. 2007. Claudin-6 and claudin-9 function as additional coreceptors for hepatitis C virus. J Virol 81:12465-71.

229. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 102:9294-9.

230. Zignego, A. L., C. Giannini, M. Monti, and L. Gragnani. 2007. Hepatitis C virus lymphotropism: lessons from a decade of studies. Dig Liver Dis 39 Suppl 1:S38-45.

ANEXO 5

ANEXO 5

Publicaciones adicionales obtenidas fruto de diferentes colaboraciones durante el desarrollo de esta tesis:

The hepatitis B virus X protein binds to and activates the NH(2)-terminal trans-activation domain of nuclear factor of activated T cells-1.
Carretero M, Gómez-Gonzalo M, Lara-Pezzi E, Benedicto I, Aramburu J, Martínez-Martínez S, Redondo JM, López-Cabrera M.
Virology. 2002; 299:288-300.

Hepatitis C virus core protein regulates p300/CBP co-activation function. Possible role in the regulation of NF-AT1 transcriptional activity.
Gómez-Gonzalo M, Benedicto I, Carretero M, Lara-Pezzi E, Maldonado-Rodríguez A, Moreno-Otero R, Lai MM, López-Cabrera M.
Virology. 2004; 328:120-30.

Increased intrahepatic cyclooxygenase 2, matrix metalloproteinase 2, and matrix metalloproteinase 9 expression is associated with progressive liver disease in chronic hepatitis C virus infection: role of viral core and NS5A proteins.
Núñez O, Fernández-Martínez A, Majano PL, Apolinario A, Gómez-Gonzalo M, Benedicto I, López-Cabrera M, Boscá L, Clemente G, García-Monzón C, Martín-Sanz P.
Gut. 2004; 53:1665-72.

A Polypodium leucotomos extract inhibits solar-simulated radiation-induced TNF-alpha and iNOS expression, transcriptional activation and apoptosis.
Ja□czyk A, Garcia-Lopez MA, Fernandez-Peñas P, Alonso-Lebrero JL, Benedicto I, López-Cabrera M, Gonzalez S.
Exp Dermatol. 2007; 16:823-9.

AM3, a natural glycoconjugate, induces the functional maturation of human dendritic cells.
Martín-Vilchez S, Molina-Jiménez F, Alonso-Lebrero JL, Sanz-Cameno P, Rodríguez-Muñoz Y, Benedicto I, Roda-Navarro P, Trapero M, Aragoneses-Fenoll L, González S, Pivel JP, Corbí AL, López-Cabrera M, Moreno-Otero R, Majano PL.
Br J Pharmacol. 2008; 154:698-708.

Epithelial-to-mesenchymal transition of peritoneal mesothelial cells is regulated by an ERK/NF-kappaB/Snail1 pathway.

Strippoli R, Benedicto I, Pérez Lozano ML, Cerezo A, López-Cabrera M, del Pozo MA.

Dis Model Mech. 2008; 1:264-74.

Hepatitis C virus NS5A and core proteins induce oxidative stress-mediated calcium signalling alterations in hepatocytes.

Dionisio N, Garcia-Mediavilla MV, Sanchez-Campos S, Majano PL, Benedicto I, Rosado JA, Salido GM, Gonzalez-Gallego J.

J Hepatol. 2009; 50:872-82.

Increased circulating pro-inflammatory cytokines and Th17 lymphocytes in Hashimoto's thyroiditis.

Figueroa-Vega N, Alfonso-Pérez M, Benedicto I, Sánchez-Madrid F, González-Amaro R, Marazuela M.

J Clin Endocrinol Metab. 2010; 95:953-62.

Expression of pituitary tumor-transforming gene 1 (PTTG1)/securin in hepatitis B virus (HBV)-associated liver diseases: evidence for an HBV X protein-mediated inhibition of PTTG1 ubiquitination and degradation.

Molina-Jiménez F, Benedicto I, Murata M, Martín-Vílchez S, Seki T, Antonio Pintor-Toro J, Tortolero M, Moreno-Otero R, Okazaki K, Koike K, Barbero JL, Matsuzaki K, Majano PL, López-Cabrera M.

Hepatology. 2010; 51:777-87.

p38 maintains E-cadherin expression by modulating TAK1-NF-{kappa}B during epithelial-to-mesenchymal transition.

Strippoli R, Benedicto I, Foronda M, Perez-Lozano ML, Sánchez-Perales S, López-Cabrera M, Del Pozo MA.

J Cell Sci. 2010; 123:4321-31.